Clinical Theriogenology
Contents
September 2013, Volume 5, Number 3

Species Abstracts

Small Animals

Production Animals

Posters

Student Case Posters

Manuscripts from the pre- and post-conference symposia and conference manuscripts not received in time to be included in the Proceedings Issue will be published in subsequent issues of *Clinical Theriogenology*.

Michelle M. LeBlanc, DVM, Dipl. ACT
A life dedicated to the study of reproduction
1954 - 2013

Michelle Mary LeBlanc, DVM, Dipl. ACT, is the 2013 recipient of the David E. Bartlett Award for Lifetime Achievement in Theriogenology. Dr. LeBlanc was a researcher, teacher, mentor, and practitioner for 36 years. Her contributions to the advancement of equine reproduction are unique and varied. A 1977 graduate of Michigan State University College of Veterinary Medicine, she spent her academic career at the University of Florida College of Veterinary Medicine and joined Rood and Riddle Equine Hospital in Lexington, Kentucky, in 2002. Practical application of her basic research guided her focus. She studied mares, stallions, foals, goats, llamas, dogs, and even elephants. She led in the development of novel research and treatment techniques in mares and foals and the invention of useful equipment. Her contributions as a teacher, administrator, and mentor defy quantification. Nominated by Dr. Audrey Kelleman, Dr. LeBlanc is a worthy recipient of this prestigious award.

Michelle grew up in Michigan with three sisters and one brother and supportive parents. Her love of animals was evident from an early age. She rode horses as a child and developed a life-long love of riding and training horses. Her last horse, BeBe, was an inspiration to her in her last months. She became a veterinarian at 24 years of age after two years of undergraduate work and three years of veterinary school. Her first job occurred by happenstance. On her way to interview for a job in Maryland, she stopped to visit Dr. Leroy Manlove who offered her a job on the spot in his "all creatures great and small" practice. After three years in practice, Dr. Maarten Drost, who remained a good friend for her entire life, recruited her to the University of Florida (UF) as a theriogenology resident. Dr. Drost sent her to Utrecht to study obstetrics, an opportunity that she characterized as an exciting time in her career development. She found like-minded individuals in the UF theriogenology group, including Dr. Victor Shille, a pioneer investigator of canine and feline reproduction. Dr. A.C. (Woody) Asbury, her mentor, immediately recognized her talents and strongly influenced her approach to research, teaching, and clinical practice as her career path began to narrow. She and Woody continued their close relationship until his passing in 2011. In this stimulating academic environment, she became a Diplomate in the American College of Theriogenologists and Assistant Professor in 1982. Ultimately, she became a Professor and Director of Equine Research in 1995.

Her early research interests took her to Washington State University to study with Dr. Lance Perryman and to Cambridge University to study with Dr. Marion Silver in 1988 and 1989. Dr. Silver's work included fetal catheterization to study the effect of medications and hormones on the fetus, a technique Dr. LeBlanc employed in her model for studying fetal maturation, induction of parturition, and fetal surgical stress in 1990. In 1995, Dr. LeBlanc wrote a monograph in *Biology of Reproduction* entitled, "Equine Fetal Maturation: The First Twenty Years. In Honor of Marion Silver". She also formed a collaborative relationship with Dr. Twink Allen during her study in Newmarket that continued throughout her career. Dr. LeBlanc returned to the University of Florida continuing the study of uterine clearance in mares leading to major improvements in treatment options for the problem mare that are now implemented routinely. Dr. LeBlanc frequently described the early 80's and 90's as "the golden age of theriogenology".

While on the faculty at UF, she received numerous awards for teaching and research, including the Carl J. Norden Distinguished Teacher in 1993. Many students, interns, and residents relate the inspiration she provided in their personal and professional lives. The list of graduate students and residents she supervised at UF reads like a list of Who's Who in Theriogenology and includes Dr. Barry Ball, Dr. Maria Cadario, Dr. Audrey Kelleman, Dr. Sara Lyle, Dr. Peter Morresey, and Dr. Dale Paccamonti, to name just a few. Many of her students have remarked on her willingness to listen as part of her positive impact on their careers. Her intense dedication to direct communication and hard work was a significant aspect of her character.

Dr. Tom Riddle enlisted her assistance in finding a theriogenologist to develop a referral service at Rood and Riddle Equine Hospital (RREH) in Lexington. In 2002, she decided that the position was right for her and she used her experience and skills with research and teaching to construct a unique amalgam of veterinary practice and research while holding an appointment as Adjunct Professor at the University of Florida. She never balked at working on the most challenging cases and enjoyed seeing the success of her

efforts at the racetrack and in the show ring. She especially enjoyed the success of Royal Delta as she won the Breeders' Cup Ladies Classic in 2011 and 2012. Dr. LeBlanc's contributions to her profession and her friends and family ended in April 2013 when she died from the complications of metastatic small bowel carcinoma.

The equine uterus was an important subject of Dr. LeBlanc's research for her entire career. She studied basic changes in endometrial histomorphology and assessed uterine changes in response to various medications and conditions. She relied on this acquired knowledge to understand the development, diagnosis, and treatment of endometritis and placentitis. Her initial work on uterine clearance mechanisms published in 1984 in *American Journal of Veterinary Research* advanced basic knowledge of the function of the myometrium. With this background, she developed a novel nuclear scintigraphy model with Dr. Lisa Neuwirth to investigate the mechanisms of uterine clearance. This model was then used to investigate the effect of different medications on uterine clearance, including her groundbreaking work on the benefit of oxytocin in uterine clearance published in *Equine Veterinary Journal* in 1994. The use of oxytocin and other ecbolics has become standard practice in the equine industry. Her focus on uterine physiology led to advances in the treatment of endometritis as detailed in *Clinical Theriogenology* in 2009.

Dr. LeBlanc arrived at RREH in 2002 after the devastating outbreak of Mare Reproductive Loss Syndrome (MRLS) in 2001. She was uniquely qualified to participate in research into the cause of MRLS based on her research in uterine pathophysiology published in the *Journal of American Veterinary Medical Association* in 1984 and her knowledge of the uterine response to medications and pathogens. In this private practice setting, she continued to study placentitis and published an in-depth paper in *Proceedings of the American Association of Equine Practitioners* in 2004 with Dr. Margo Macpherson and Dr. Pete Sheerin. She worked with a team of researchers to determine the root cause of MRLS as published in *Journal of the American Veterinary Medical Association* in 2004 and continued to study the effect of placentitis on the fetus published in *Theriogenology* in 2007 with Dr. Audrey Kelleman and others.

In other research during private practice, she, Dr. J. Magsig, and Dr. A.J. Stromberg refined the technique of low volume uterine lavage for evaluation of sub-clinical endometrial conditions as detailed in *Theriogenology* in 2007. She used endoscopic techniques to assess the endometrium and uterine function. Results of her work with the scanning electron microscope to investigate cellular function of the endometrium were published in abstract form in *Proceedings of the 10th International Symposium on Equine Reproduction (ISER)*. One of the subjects of her work with Dr. Robert Causey was the interaction of the uterus with biofilms and the effect of N-acetylcysteine and other mucolytics on uterine function with the initial publication accepted just prior to her death.

Dr. LeBlanc's interest in the equine uterus naturally evolved into studies of fetal stress and treatments to improve fetal survival. From this work, she established protocols for clinical assessment, identification, and treatment of the compromised equine fetus published in the *Equine Veterinary Journal* in 1997. She developed and patented a colostrometer in 1985 still in use today as an aid in measuring colostrum immunoglobulin concentration to predict its value to the equine neonate. Her research in the early 1980's describing the relationship between colostral immunoglobulin concentration and absorption by foals was published in *Journal of Reproduction and Fertility* in 1987. Later, she described factors influencing passive transfer of immunoglobulin published in *Journal of the American Veterinary Medical Association* in 1992. Her interest in fetal and neonatal viability was a driving force for her placentitis research. In 2010, she published an update on ascending placentitis in the mare in *Reproduction of Domestic Animals Supplement*. She collaborated in placentitis research with Dr. Dale Paccamonti, Dr. Steeve Giguere, and others on the relationship between infection, inflammation, and premature parturition in mares with experimentally induced placentitis. She had begun work with Dr. David Horohov studying several inflammatory cytokines as possible markers of the early development of placentitis. Her work with fetal stress resulted in improved survival of foals delivered from sick mares and mares with placentitis.

Dr. LeBlanc has received awards recognizing her meritorious work from students, practitioners, industry, academics, and research peers. She received the Lifetime Achievement Award from the Florida Association of Equine Practitioners in 2011. The Florida Thoroughbred Farm Managers industry group honored her with its Carry Back Award in 2011. Her alma mater Michigan State University granted her the Distinguished Veterinary Alumnus Award in 2007. She was named the Theriogenologist of the Year in 2000

by the American College of Theriogenologists. She received the World Equine Veterinary Association (WEVA) Lifetime Achievement Award in 2011, which exemplifies her impact on international equine veterinary knowledge. The WEVA Lifetime Achievement Award honors clinical research, basic and applied research, and teaching. She is only the second recipient of this prestigious award, the first being Dr. Peter Rossdale in the United Kingdom.

Dr. LeBlanc generously shared her knowledge and ideas in many ways and welcomed constructive debate in all forums. She traveled internationally speaking to many equine veterinarians and industry professionals about new methods for improving equine health. The list of international groups she addressed is long and prestigious. She spoke to the Society for Italian Equine Veterinarians, which awarded her honorary membership, and to several Japan Racing Association Symposiums. Her textbook contributions have provided innovative information to many students and veterinarians. She co-authored or contributed chapters to at least twelve textbooks. Her individual mentoring of students, interns, and residents provided a major contribution to the dissemination of equine health. She served on the boards and committees of many organizations to ensure the highest quality equine research. The Havemeyer Foundation was a major supporter of Dr. LeBlanc's research and she organized several of the in-depth Havemeyer Workshops including a unique comparison of uterine infection in women and mares in 2004. She served as a member of the Gluck Equine Research Veterinary Advisory Board. She served on boards and committees for the Society for Theriogenology (SFT), the American Association of Equine Practitioners (AAEP), the International Symposium for Equine Reproduction (ISER), and the American College of Theriogenologists (ACT). Many other professional organizations benefitted from her insight and effort. Dr. LeBlanc served as president of the ACT in 1997-98 and on the Board of Directors of AAEP from 2004-07. She participated in AAEP seminars, organizing the first Focus In-depth Seminar, SFT programs as chair and presenter, and equine industry meetings to educate clients and veterinarians about advancements in equine reproduction. She assisted in developing two recurring symposiums, first, in Kentucky as the Bluegrass Symposium, then, in California as the West Coast Equine Reproduction Symposium, to help raise funds for graduate student participation in ISER. Her contributions to these organizations provide a record of her enormous positive influence on her profession both nationally and internationally.

Her impact on the field of equine reproduction through her research accomplishments is permanent. Those she mentored in myriad ways during her 36 years as a theriogenologist are a significant part of her legacy. Her life included her beloved husband, Kevin Anderson, who passed away in 2011 from brain cancer, her dear dogs, and her joy for all outdoor activities especially cycling and running. She loved the beach and spent many happy hours at Amelia Island or St. Augustine Beach. She enjoyed a cup of good coffee or hot tea as she read for pleasure an astonishing number of books, both fiction and non-fiction. Planning the gardens at her Florida and Kentucky homes was a pleasant challenge for her. Her love of Florida basketball is legendary as evidenced by her request, perhaps tongue-in-cheek, to have Florida-Kentucky basketball tickets as part of her compensation package at RREH. Many of her students remarked on her femininity as she worked in a male dominated profession, wearing her elegant earrings and well-tailored clothes, presenting a smart, in so many ways, role model for young women veterinarians. We will always remember her wonderful smile and her willingness to tackle any problem head on.

Thank you, Michelle.

This essay was written by Carol McLeod based on her friendship with Michelle, conversations with Michelle over many years, reference to Michelle's curriculum vitae, and remembrances by Michelle's friends. Any errors are the sole responsibility of Carol McLeod, for which apology is made and correction requested.

Behavioral principles of keeping cattle calm

Temple Grandin
Department of Animal Science, Colorado State University, Ft. Collins, CO

Calm animals are much easier to handle than fearful, frightened animals. If cattle or horses become agitated, they should be given 20 to 30 minutes to calm back down. Yelling is highly distressful to livestock and cattle had greater heart rates in response to people yelling compared to the sounds of gates slamming.

Cattle will often balk and refuse to walk through a chute when they see distractions. Some of the most common distractions that cause balking are: contrasts between different types of flooring, reflections on shiny metal or water, seeing moving people up ahead vehicles parked by a chute, dangling chains, and coats on fences. Removing distractions will improve animal movement. Animals that are out in bright sunlight will often refuse to enter a dark building. They will often enter more easily if they can see daylight through the building. Installation of white translucent skylights to let in daylight is also effective. The use of an enclosed breeding box will help keep cattle calmer. To facilitate entry, it should have a small window in the front. It is essential that there are no distractions in front of the window such as vehicles or people. Nonslip flooring is essential. Animals become agitated and fearful when they slip. The behavioral signs that indicate that horses and cattle are becoming fearful are visible eye white and swishing tails. In horses, sweating, trembling and nostrils flaring may also be visible. These behavioral indicators often provide an early warning before an animal kicks, bites, or lashes out at people.

Another important principle for keeping beef cattle calm is moving small groups through the crowd pen that leads to the single file chute. Fill the crowd pen half full and use following behavior. Wait until there is space in the single file chute before filling the crowd pen. This enables cattle to pass through the crowd pen and follow the leader into the single file chute.

Selected references

Grandin T: Livestock handling and transport. 3rd ed. Wallingford(UK): CABI Publishing; 2007.
Grandin T, Deesing M: Humane handling and transport. North Adams(MA):Storey Publishing; 2008.
Grandin T: Factors that impede animal movement at slaughter plants. J Am Vet Med Assoc 1996;209:757-759.
Grandin T: Handling methods and facilities to reduce stress on cattle. Vet Clin North Am Food Anim Pract 1998;14:325-341.

Commercial dog breeding: implications for animal well-being

Candace C. Croney
Department of Animal Sciences, Purdue University, West Lafayette, IN

Abstract

Commercial breeding of dogs is an emotionally laden, contentious and complex topic. Opposition to "puppy mills" appears to have increased in the past decade in the U.S., with a growing number of legislative initiatives aimed at regulating such operations. Concerns about dogs reared for commercial breeding purposes vary. However, they range from physical impingements on dog well-being as a function of their nutrition, genetics, housing, husbandry practices, reproductive and general health to behavioral wellness issues. Recent efforts to establish standards for humane care of commercially reared dogs have focused primarily on addressing sanitation and physical health issues. However, relatively little is offered in the way of mandating conditions that support behavioral well-being in these dogs, an oversight that is particularly problematic given that the intended purpose of the animals is for sale as companions. Behavioral problems in commercially reared dogs may stem from many of the same sources as the physical welfare issues (genetics, environment) and may manifest in undesirable behaviors such as excessive vocalization, anxiety and fearfulness, destructive behavior and aggression.

As efforts to regulate commercial dog breeding continue, there are likely to be increased opportunities for veterinary oversight of the care management and well-being of the animals. Improved incorporation of expertise in theriogenology, animal behavior and welfare will be essential to improving the quality of life experienced by dogs used for commercial breeding purposes.

Keywords: Animal welfare, dog breeding, behavior,

Introduction

Animal welfare is a highly contentious, complex component of contemporary food animal production which receives a significant amount of media coverage in the U.S. and abroad. Unsurprisingly, concerns about animal treatment extend to those used for other purposes, including for research, teaching, sport/entertainment and companionship. The commercial breeding of dogs, commonly referred to as "puppy mill" breeding, currently faces public scrutiny and sentiment not unlike that directed at intensive confinement farming of food animals. However, high volume confinement production of dogs is even more emotionally laden in part because of the cultural connotations associated with human-dog interactions in western developed nations, and because many people's primary relationships with animals in such countries stem from frequent, positive interactions with companion animals. Given that over 36% of US households own dogs,[1] commercial breeding of dogs, particularly under conditions that appear to harm them, evokes strong reactions by the U.S. public. Public perceptions of such operations tend to be highly negative although many people continue to purchase animals from uncertain origin (parking lots, for example) or from businesses known to source animals from commercial pet breeding operations. Little information exists relative to understanding the apparent discrepancies between public perceptions about commercial dog breeding and public purchasing and voting behavior relative to commercially bred dogs.

Commercial dog breeding is poorly received by many as it is often thought to exacerbate existing welfare problems associated with overpopulation of cats and dogs in shelters and rescues, thus contributing to already high rates of companion animal relinquishment, abandonment and euthanization, and the draining of financial resources of humane organizations. It is estimated that there are more than 78 million pet dogs in U.S.;[2] of these, approximately four million are euthanized each year, with behavioral problems cited as the leading cause of relinquishment to shelters.[3] Such problems are potentially worsened by flooding the market with animals that may or may not be successfully homed, or that may be returned due to physical or behavioral health problems. Consequently, scathing criticism of commercial dog breeding is common and has resulted in polarized, fractious debates, and ongoing attempts to alter, limit or entirely eliminate commercial dog breeding operations.

Understanding animal welfare in the context of commercial dog breeding

Although the term "animal welfare" carries multiple connotations for different people, it fundamentally pertains to value-laden notions about animal quality of life. According to Broom,[4] welfare is the state of the animal in regards to its attempts to cope with the demands placed on it. Concerns about animal welfare are rooted in the belief that people have obligations to maintain an acceptable standard for the care and well-being of animals we maintain and utilize for different purposes. The challenge, of course, is to agree upon what that acceptable standard looks like. For some people, animal welfare is "just good husbandry," in other words, it simply entails providing for animals' basic needs for food, water, shelter and veterinary care. However, there is growing consensus that that for an animal to do well, both its physical and behavioral health must be addressed.[4,5] In other words, animals must feel well, function well and whenever possible be permitted to exhibit natural behaviors commensurate with its adaptations. These conceptions are captured by the Five Freedoms, outlined by the Brambell Committee in the United Kingdom in 1965,[6] which have now become the basis for many animal welfare initiatives in developed western nations. The Five Freedoms correspond to the three basic concepts of welfare.[5] In essence, they are aimed at ensuring that farmed animals are not deprived of water and food and thus are not malnourished; they also promote the idea that animal health is crucial--animal should be free from preventable diseases, injury and functional impairment. Further they encode the notion of animals having behavioral needs which are critical to their well-being. Thus, they should not have their behavior restricted without good justification and also should not experience unnecessary mental suffering.

Different stakeholders, though, may disagree in their prioritization of these dimensions of animal welfare. For example, a dog breeder who emphasizes the physical aspects of well-being may focus primarily on ensuring freedom from malnourishment and from preventable disease and injury, whereas an applied ethologist may consider the capacity of animals to behave normally to be of utmost importance. Regardless of which conception of welfare is chosen or which criteria are prioritized, the challenge for commercial dog breeders is to clearly articulate and demonstrate that their animals are maintained according to standards that permit them to retain a social license to operate.

Regulation of "puppy mills"

In the past decade, attempts to regulate commercial dog breeding operations have increased dramatically. While the Animal Welfare Act has mandated standards for the care of dogs and cats other kinds of bred for commercial sale since 1966, breeders who directly to the public are not covered, increasing the risks that animals can be maintained in subpar conditions in puppy mills with little protection other than that offered by state anti-cruelty statutes. However, compounding this issue is the problem of defining such an operation in the first place. There is little consensus on whether a "puppy mill" is created as a function of the number of animals maintained for breeding, the total number of animals maintained, the number of litters produced annually, or as a function of the living conditions, management and quality of care provided to the animals. For example, in 2008, Virginia's House Bill 538 defined a commercial breeder as "a person who, during any 12-month period, maintains 30 or more adult female dogs for the primary purpose of the sale of their offspring as companion animals."[7] However, initially the number of animals proposed was lower (20 adult females) and this number varies across states with established standards. Further, localities are permitted to adopt ordinances allowing more dogs.[8] Moreover, state standards for the humane care of dogs categorized as coming from a commercial breeding operation vary widely, with some states establishing requirements for housing, sanitation, nutrition and veterinary care, while others do not clearly specify standards in these areas. Few if any, appear to mandate conditions relative to behavioral well-being of the animals

Welfare issues associated with commercial breeding implications for theriogenology

As is the case for food animal production today, a number of welfare issues can be encountered in commercial dog breeding that influences animals' physical and behavioral well-being. These relate to the quality of housing provided to the animals, including space allocation, flooring, lighting, temperature,

ventilation and air quality. In addition, the quality of nutrition and veterinary care provided are important factors. Criteria for, timeliness and method (s) of euthanization must also be considered.

The reproductive management of the animals is a key consideration and an area of major criticism for opponents of such breeding operations. Concerns here include breeding of dogs without due consideration for the criteria on which animals are selected to reproduce (beyond aesthetics) and without sufficient expertise to support, evaluate and document the reproductive and overall health of individual animals and with consideration for different breeds. In addition, expertise is needed to determine appropriate ages for breeding animals, and the number and quality of litters that can reasonably be expected to be produced in a given time frame without undue physical distress and deterioration to the breeding animals and their offspring. Appropriate and timely intervention schemes must also be derived that better reflect current scientific discoveries in theriogenology.

Initial attempts to improve animal well-being relative to reproductive health can be noted in several of the provisions under existing "puppy mill bills" that have been passed and those currently being proposed. For example, VA's HB 538 requires "annual certification by a licensed veterinarian that the dog is in suitable health for breeding" and limits the age at which dogs can be bred.[7] As similar legislation continues to be proposed and passed in other states it is likely that increased demand for veterinarians, particularly those specializing in theriogenology, will be created by breeders attempting to come into compliance.

The behavioral well-being of commercially bred dogs can be equally problematic, and as noted earlier, is often entirely overlooked or minimally attended to in established standards. Appropriate genetic selection of animals that can tolerate commercial rearing conditions is rarely discussed.[9] Likewise, selection of breeding animals that are behaviorally sound is insufficiently discussed. Since the leading cause of pet animal relinquishment is behavioral problems, it is both ethically and scientifically problematic to breed and commercially distribute dogs at risk for or currently exhibiting behavioral abnormalities given that the intention is for these animals is to live harmoniously with people for the duration of their lives. Further, there is high propensity for suffering if animals maintained for commercial breeding do not receive adequate attention to their needs for enrichment, normal social interactions with conspecifics, positive human-animal interactions, appropriate socialization and sufficient exercise.

Failure to ensure that these criteria are met can potentially place dogs result in unpleasant states such as boredom, frustration and chronic arousal/distress.[10] Studies on the life- long physical and behavioral impairments of animals experiencing distress in utero and improper or undue neonatal stress provide strong impetus to scrutinize commercial breeding operations in regard to care and management practices that may result in maternal stress in breeding bitches that in turn negatively impacts puppies via chronic activation of the hypothalamic pituitary adrenal axis.[11] Impaired learning, chronic arousal and increased sensitivity to stress-inducing stimuli that manifests in fearfulness and aggression to people and other animals are likely to be poorly tolerated by dog owners. Other problematic behaviors that may be facilitated by improper genetic selection and environmental management of commercially bred dogs include excessive vocalization, house soiling, and destructive behaviors.

Dogs that are not carefully genetically selected and managed are therefore at heightened risk for mistreatment, relinquishment, abandonment or euthanasia. High volume commercial breeding operations that produce animals that are behaviorally unsound are likely to face continued public ire, increased regulation and further deterioration of their social license to operate.

As efforts to regulate commercial dog breeding continue, there are likely to be increased opportunities for veterinary oversight of the care management and well-being of the animals. In absence of societal consensus on whether commercial breeding of dogs should occur at all, better incorporation of expertise in theriogenology, animal behavior and welfare is essential to improving the quality of life experienced by dogs currently used for commercial breeding purposes.

References

1. American Veterinary Medical Association: U.S. pet ownership & demographics sourcebook; 2012.

2. American Pet Products Association: National pet owners survey 2011-2012; 2012.
3. Scarlett JM, Salman MD, New JG; et al: Reasons for relinquishment of companion animals in U.S. animal shelters: selected health and personal issues. J Appl Anim Welf Sci 1999;2:41-57.
4. Broom DM: The scientific assessment of animal welfare. Appl Anim Behav Sci 1988;20:5-19.
5. Fraser D; Weary DM, Pajor EA, et al: A scientific conception of animal welfare that reflects ethical concerns. Anim Welf 1997;6:187-205.
6. Webster AJF: Farm animal welfare: the five freedoms and the free market. Vet J 2001;161:229-237.
7. Va. Code Ann. § 3.2-6507.2(2) (Repl. Vol. 2008).
8. University of Richmond Law Review: Animal Law (2009 Annual Survey of Virginia Law);2009. http://lawreview.richmond.edu/animal-law/. Accessed, April 1, 2013.
9. Meaney M: Epigenetics and the biological definition of gene x environment interactions. Child Devel 2010;81:41-79.
10. Francis DD, Diorio J, Plotsky PM, et al: Environmental enrichment reverses the effects of maternal separation on stress reactivity. J Neurosci 2002;22:7840-7843.
11. Francis D, Meaney M: Maternal care and the development of stress response. Curr Opin Neurobiol 1999;9:128-134.

A brief overview of reproduction in common rodent pocket pets

Angela M. Craig
Department of Research Animal Resources, University of Minnesota, Minneapolis, MN

Abstract

The aim of this paper is to provide a brief overview of the reproductive characteristics of six common small rodent species typically seen in pocket pet practice and used as animal models in research studies. These species are the mouse, rat, gerbil, hamster, chinchilla, and guinea pig.

Keywords: Rodent, pocket pet, laboratory animal, reproduction

In private practice, knowledge of normal characteristics, similarities and differences among the species, and common reproductive problems can aid the practitioner in providing services requested by clients who present their rodent pets for annual examinations, sexing, litter prevention education, and interventions necessary during abnormal reproductive events. From a biomedical research perspective, understanding the reproductive processes in common animal models guides scientists and laboratory animal veterinarians as they develop studies and directs breeding colony management decisions.

Mouse (*Mus musculus*)

Mice may be less commonly presented in small animal practice than other pocket pets, but this species is the workhorse of biomedical research. The mouse is the most commonly used mammalian laboratory animal worldwide. Mice breed readily, are genetically well-characterized, and have a short generation time. Managing mouse breeding in laboratory animal facilities is a standard component of colony management.

Males can be differentiated from females by examining the anogenital distance. This distance from the anus to the genital opening is 1.5-2 times greater in the male than the female.[1] Both sexes have an external urinary papilla, though it often appears larger in the male. With gentle digital pressure on either side of the papilla, the penis of the adult male can be everted. Males have an os penis. In the perineal region, the female has paired clitoral glands and the male has paired preputial glands. Both types of glands can develop secondary infections causing visible swelling.[2] Females have five pairs of mammary glands. Males do not have visible nipples.

Female mice are continuously polyestrus and are spontaneous ovulators with a cycle of 4-5 days. They are most receptive to breeding during the dark phase of their light cycle.[3] Males are vigorous breeders and can successfully impregnate small harems of females. Following mating, the male leaves a copulatory plug in the female's vagina. The plug is a concretion of secretions from the seminal vesicles and coagulating gland.[4] It is believed to help retain the ejaculate in the vagina and prevent breeding of the receptive female by another male. The plug remains in the vagina for up to 24 hours before falling out. To have the best chance of finding one in *situ*, the female should be checked in the early morning following overnight exposure to a male. The presence of the plug confirms that mating has occurred, but does not guarantee pregnancy.

Three pheromone-based effects are well-described in the mouse. They are the Whitten, Bruce, and Lee-Boot effects.[5] When exposed to a male or his scent, 40-50% of female mice housed together will cycle into estrus synchronously within three days. This is the Whitten effect. A pregnant mouse exposed to a strange male mouse or his scent during the first one to four days of pregnancy will have failure of implantation. This is the Bruce effect. The Lee-Boot effect describes how females housed together in the absence of a male will have false pregnancy or stop cycling altogether. With mindfulness of these effects, breeding managers attempt to achieve optimum reproductive performance in their colonies. A typical breeding strategy in mouse colonies is to have one male service a harem of one to three females. In a laboratory setting, a reason for having one male housed continuously with a single female is that he can take advantage of the post-partum estrus which occurs 14-24 hours following parturition.[6]

Mice have a bicornuate uterus and are capable of carrying litters of one to 12 young.[5] Mouse gestation is 19-21 days.[5] By late gestation females assume a pear-shaped appearance as the young distend the caudal abdomen. Females give birth to altricial young. Newborn pups are completely hairless and have sealed eyes and ears. Their skin is bright red at birth and develops into a light pink color over the next few days. Post-natal pup development is well described in the literature and they can easily be aged by their developmental state. Most are ready to wean at 21 days. In some laboratory strains, the pups mature more slowly and require later weaning at up to 28 days.[1] Mice become sexually mature by seven to eight weeks.[5]

Adult male mice can be aggressive to other males and can be suspicious fathers. Infanticide and cannibalism can occur if a male mouse is reintroduced to a female with a litter whether or not he fathered the pups. It is important not to disturb the early postpartum female and breeding colony staff will often wait at least three days after parturition before entering the cage.

Dystocia occurs regularly in large breeding colonies.[5] The prevalence may be due to the high number of pregnant animals, and the wide variety of strains, many of which are selected for traits unrelated to reproductive success. Similar to other species, causes of dystocia are an overlarge pup, two pups descending at the same time, maternal anatomic abnormalities, and uterine inertia. Unfortunately, oxytocin administration is rarely successful in dystocic dams. Supportive care, including fluids, warmth, analgesia, nutritional supplementation, and lubrication of the vaginal canal, is believed to have a greater chance of bringing the female to successful parturition.[7] In a laboratory setting, if the mother cannot be saved and the pups are valuable, cesarean section with euthanasia of the dam may be undertaken to prevent loss of the young. The living pups can be fostered onto a lactating, receptive mother.

Mammary neoplasia in mice is most commonly adenocarcinoma. By the time it is detected, metastasis to the lungs may already have occurred.[8]

The prime breeding period for mice in laboratory conditions is seven to eight months long and is it common to replace breeders that are older than one year or have gone 60 days since their last pregnancy. Ovariohysterectomy and castration of mice are performed occasionally in pet practice, and these, in addition to ovariectomy, are well-described in the literature due to their importance for specific research studies in which reproductive hormones must be manipulated.

Rat (*Rattus norvegicus*)

As intelligent, social, and tractable animals, rats are popular pocket pets and common research models. Like mice, they have been used extensively in research and much is known about their reproductive physiology and behavior.

As in mice, the anogenital distance is used to differentiate males from females. Adult male rats have pendulous testicles. They can draw them into the abdomen via the inguinal canal, which remains open throughout life. Males have an os penis. Females have six pairs of mammary glands.[9] Males have no nipples.

Females are continually polyestrus, and are spontaneous ovulators with a four to five day cycle. The estrous cycle is light sensitive and if exposed to constant light, rats can develop persistent estrus and polycystic ovaries.[10] Optimal lighting conditions to promote appropriate cycling are described in the literature. After breeding, the male leaves a copulatory plug in the vagina.

The Bruce effect does not occur in rats. The Whitten effect is not believed to occur. The Lee-Boot effect occurs, but is much less pronounced in rats when compared to mice.[11]

Rats have a 21-23 day gestation.[3] They give birth to three to 18 pups.[11] Rat pups are hairless and helpless at birth. Rat dams are nurturing mothers and male rats, if kept with the mother as a pair, will help care for the pups. Rat mothers will nurse each others' pups and if kept in groups, mothers may share a nest.[10,12] In contrast to mice, neither sex is particularly prone to infanticide or cannibalism of the young. Rat pups wean at 21 days.[11] They are sexually mature by 40-60 days.[11]

Mammary neoplasia is common in both sexes. In most cases it is benign fibroadenoma. Even when rats present with very large mammary masses, removal is relatively simple. Unfortunately, additional masses may occur along the mammary chain during the remainder of the animal's life. Spay of

the female may be preventative.[13] Both ovariohysterectomy and castration are well described in the literature.

Mongolian gerbil (*Meriones unguiculatus*)

Male gerbils are easy to sex even at a young age due to their darkly pigmented scrotum. The anogenital distance is also reliable. Both sexes have a ventral midline scent gland, but in adult males it is well-developed. Females have four pairs of teats.

Male gerbils become sexually mature by 70-84 days of age.[14] Vaginal opening occurs at 40-60 days, but females may not achieve sexual maturity until 30 days later.[14] Breeding pairs are monogamous, and if the male dies the female may not accept another partner. Females are continually polyestrus, and are spontaneous ovulators with a four to six day cycle. Mating generally occurs during the evening hours. Females have a postpartum estrus.[15] If no young from a previous litter are being nursed, gestation is 24-26 days.[16]

Like mice and rats, gerbil mothers should not be disturbed immediately following parturition. Litters contain three to eight young.[17] Pups are dependent on the mother until weaning at 21-28 days. Bonded pairs will share care of the young.[18]

Reproductive disorders are relatively uncommon in gerbils in comparison to other species. Cystic ovaries are common in old females. Cysts can form on one or both ovaries and reproduction is adversely affected.[19] For animals that present with severe abdominal distension and dyspnea, ultrasound-guided cyst aspiration will reduce intrabdominal pressure temporarily, but only ovariohysterectomy is curative.

Golden or Syrian hamster (*Mesocricetus auratus*)

Hamsters, like the rodents already described, can be sexed by anogenital distance. Males have pendulous testicles in adulthood which gives them a rounded perineal silhouette when viewed from above. Females have an angular, tapering perineal silhouette. Both sexes have bilateral flank glands, but females do not have the androgen stimulation needed to make them apparent. When males are sexually stimulated, the glands become moist with secretions.[17] Females have six to 17 teats.[20]

Sexual maturity occurs by six to eight weeks of age.[21] Females are continuously polyestrus, and are spontaneous ovulators with a four day cycle. They produce a creamy, white, strong-smelling vulvar discharge around day two of estrus.[21] For practitioners unfamiliar with hamsters, this can be mistaken for pus, but pyometra is rare in this species. Vaginal cytology is not a reliable way to determine the stage of estrus because sloughed cells collect within the paired vaginal pouches.[17]

Hamsters can be aggressive to one another. When mating is desired, the female should be added to the male's enclosure or both should be placed in a neutral enclosure. Close observation is necessary and if the female attacks the male, they should be separated immediately to prevent life-threatening damage. They can be reintroduced later when she is at a more receptive phase of her cycle. When she is receptive, the female will exhibit lordosis and allow the male to breed her multiple times within a short period. After mating they should be separated.

The gestation period is 15-18 days.[22] Females have an infertile postpartum estrus.[23] Litter size ranges from four to 12 pups.[22] The pups are altricial. New mothers, especially primiparous dams, are highly prone to litter desertion, infanticide, and cannibalism. Following parturition, they should be left undisturbed for one to two weeks to prevent these problems. Hamsters will not accept orphan young and may kill both their own young and the orphans if an attempt at fostering is made.[23]

When disturbed, hamster dams may move their litter around the enclosure to new nesting sites, using their cheek pouches to carry the young. This can appear as cannibalism to the untrained eye. Clients may report mysteriously reappearing litters after they thought the mother had eaten the young. The young are weaned at 21-25 days.[24] They should be separated from the mother shortly thereafter.

Chinchilla (*Chinchilla lanigera*)

Chinchillas can be sexed using anogenital distance. The external appearance of the genitalia can be deceiving. The female has a cone-like urinary papilla which can be mistaken for a penis and prepuce.

This papilla is also called the clitoris or urethral cone. To ensure correct sexing, gentle digital pressure can be used to attempt to evert a penis from the papilla. Females have three pairs of mammary glands. Vaginal closure membranes are present except during estrus and parturition.[25] In males the inguinal canals are open throughout life. There is no true scrotum.

Chinchillas are sexually mature by eight months of age, but the onset of sexual maturity ranges from two to 14 months.[25] Female chinchillas are seasonally polyestrus, and are spontaneous ovulators with an estrus cycle of 30-50 days.[25] During estrus no swelling of the vulva is apparent, but the perineal skin tone flushes to a dark red.[26] In captivity two litters may be born between November and May.[27] Breeding strategies in chinchillas include pairing or polygamous groups with one male to a harem of six to 12 females. Females may not tolerate the presence of the male after parturition. Copulatory plugs are common after mating.

Chinchilla gestation is long at an average of 111 days, allowing for greater neonatal development and the birth of precocious young.[25] Females give birth in the morning, eat their placenta, and nurse their young while standing erect.[26] Litter size is one to five pups, with two being average.[25] Pups can eat solid food within a week of birth, but they typically wean from dam's milk at six to eight weeks of age.[25]

A chinchilla that is in labor for more than four hours or is attempting to give birth after noon may be in dystocia. Other signs include restlessness, distressed vocalization, and continual cleaning of the genital area.[25] Causes include uterine inertia, malpositioned fetuses, or oversized fetuses. Cesarean section should be performed to relieve the dystocia. It is generally well-tolerated if the dam is in good condition when presented.

Chinchillas may not produce milk for up to 12-72 hours after parturition. If necessary, oxytocin can be administered to promote milk-letdown. If unsuccessful, the young must be hand-raised.

In males, the primary reproductive concern is a fur ring on the penis. Males may present with paraphimosis, straining to urinate, and excessive cleaning of the penis.[28] When examined, there will be a yarn-like ring of compacted fur around the penis. It should be well-lubricated and cut away. A hooked suture scissors works well to remove fur rings. Fur rings occur in both sexually active and inactive males, but the condition is much more common in breeding animals. The examination of the penis for a fur-ring should be part of every annual examination and owners can be taught to check for the condition so the ring can be removed before damage occurs.

Guinea pig (*Cavia porcellus*)

Male guinea pigs are called boars and the females are sows. Males have visible scrotal pouches and large testes that can be drawn up into the abdomen through the inguinal canal, which is open throughout life. Gentle abdominal pressure will help drop the testes into the scrotal pouches. The penis can be extruded with gentle digital pressure. The boar has an os penis. Internally, the seminiferous vesicles are extensive and can extend 10 cm cranially into the abdomen.[29] Due to their size and texture, they could be misidentified as a uterus.[30] Adult boars have a scent gland on their lower back. In aged boars it can be become large and encrusted with secretions.

Visually, females have a Y-shaped appearance to the genital area. Like the chinchilla, they have vaginal membranes which cover the opening except during breeding and parturition.[30] Both sexes have a single pair of nipples on the caudal abdomen.

Females are sexually mature by two months of age and males are mature by three months, though they will begin mounting behavior as early as one month of age.[31] Females are polyestrus, and are spontaneous ovulators with an estrous cycle of 15-17 days.[30] An estrus sow will exhibit lordosis. Males leave a copulatory plug after mating.

Gestation is long, at 59-72 days, and this period allows for greater development of the young.[32] Like chinchillas, guinea pigs eat their placenta. They have a postpartum estrus.[31] There are generally two to five young per litter.[31] They are precocious at birth, but cannot fend entirely for themselves. Sows can be passive caregivers, allowing nursing to take place, but not actively seeking out their young.[33] They can eat solid food within a week of birth, but pups may nurse for up to three weeks. They are weaned by 14-21 days.[30]

Dystocia is common in sows which are bred for the first time after six to seven months of age.[30] Prior to parturition, the pubic symphysis separates with a gap of up to 25 mm or more. If this does not occur early in life, the pubis cannot open as far or is fused.[30] Subsequently, the older primagravid sow cannot pass her young and develops dystocia. Other causes of dystocia include obesity, large fetal size, and pregnancy toxemia. Normal delivery of guinea pigs is rapid, with pups coming out within a few minutes of one another. Dystocia should be suspected in a sow that becomes depressed or develops bloody to dark brown vulvar discharge. A cesarean section is warranted, but if the sow presents severely debilitated from long hours of laboring the prognosis is guarded even with intervention.

Sows are also prone to pregnancy toxemia, vaginitis, and mastitis.[34] Clinical signs and treatment strategies are described in the literature.[34] Ovarian cysts are very common in middle-aged to old sows.[35]

Males experience a condition similar to vaginitis in the sow in which their genital area becomes impacted with bedding and fecal material.[34] Boars may develop orchitis or epididymitis following sexual contact with a sow. These problems also occur through trauma or hematogenous spread of Bordetella or other common bacteria.[34]

This paper is not exhaustive. Information about internal anatomy, copulatory and maternal behavior, medical and surgical treatments, and pathology are well-described in the literature. Excellent texts, written for both laboratory animal veterinarians and exotic animal practitioners, exist and serve as valuable resources. Many of these are referenced as part of this overview and numerous others, while not mentioned directly, have contributed greatly to the vast body of information on small rodent reproduction.

References

1. Hrapkiewicz K, Medina L: Clinical laboratory animal medicine: an introduction. 3rd ed. Ames (IA): Blackwell Publishing; 2007. p. 43-44.
2. Plikus MV, Sundberg JP, Chuong CM: Mouse skin ectodermal organs. In: Fox JG, Barthold SW, Davisson MT, et al, editors The mouse in biomedical research. 2nd ed. Vol. 3. Normative biology, husbandry, and models. Burlington: Academic Press; 2007. p. 711.
3. Bennett JP, Vickery BH: Rats and mice. In: Hafez ESE, editor. Reproduction and breeding techniques for laboratory animals. Philadelphia: Lea and Febiger; 1970. pp. 299-303.
4. Rugh R: The mouse: its reproduction and development. Minneapolis: Burgess Publishing Company; 1968. p. 45.
5. Suckow MA, Danneman P, Brayton C: The laboratory mouse. Boca Raton: CRC Press; 2001. pp. 29-30.
6. Jacoby RO, Fox JG, Davisson M: Biology and diseases of mice. In: Fox JG, Anderson LC, Loew FM, et al, editors. Laboratory animal medicine. 2nd ed. New York: Academic Press; 2002. p. 50.
7. Narver HL: Oxytocin in the treatment of dystocia in mice. J Am Assoc Lab Anim Sci 2012;51:10-17.
8. Percy DH, Barthold SW: Pathology of laboratory rodents and rabbits. 3rd ed. Ames (IA): Blackwell Publishing 2007. p. 116-117.
9. Lohmiller JJ, Swing SP: Reproduction and breeding. In: Suckow MA, Weisbroth SH, Franklin CL, editors. The laboratory rat. 2nd ed. Boston: Elsevier Academic Press; 2006. p. 152.
10. Maeda K, Ohkura S, Tsukamura H: Physiology and reproduction. In: Krinke GJ, editor. The laboratory rat. San Diego: Academic Press; 2000. p. 160-173.
11. Sharp PE, LaRegina MC, Suckow MA: The laboratory rat. Boca Raton: CRC Press; 1998. p. 15-17.
12. Hanson AF, Berdoy M: Rats. In: Tynes VV, editor. Behavior of exotic pets. Ames (IA): Wiley-Blackwell; 2010. p. 105.
13. Hotchkiss CE: Effect of surgical removal of subcutaneous tumors on survival of rats. J Am Vet Med Assoc 1995;206:1575-1579.
14. Norris ML, Adams CE: Vaginal opening in the Mongolian gerbil, *Meriones unguiculatus*: normal data and the influence of social factors. Lab Anim 1979;13:159-162.
15. Marston JH, Chang MC: The breeding, management and reproductive physiology of the Mongolian gerbil (*Meriones unguiculatus*). Lab Anim Care 1965;15:34-48.
16. Norris ML, Adams CE: Mating post partum and length of gestation in the Mongolian gerbil (*Meriones unguiculatus*). Lab Anim 1981;15:189-191.
17. Lennox AM, Bauck L: Basic anatomy, physiology, and clinical techniques. In: Quesenberry KE, Carpenter JW, editors. Ferrets, rabbits, and rodents: clinical medicine and surgery. 3rd ed. St. Louis: Elsevier/Saunders; 2012. p. 341-342.
18. Marston JH: The Mongolian gerbil. In: Universities Federation for Laboratory Animal Welfare: The UFAW Handbook on the care and management of laboratory animals. 5th ed. New York: Churchill Livingstone; 1976. p. 263-274.
19. Norris ML, Adams CE: Incidence of cystic ovaries and reproductive performance in the Mongolian gerbil, *Meriones unguiculatus*. Lab Anim 1972;6:337-342.

20. Bivin WS, Olsen GH, Murray KA: Morphophysiology. In: Van Hoosier GL, McPherson CW, editors. Laboratory hamsters. Orlando: Academic Press; 1987. p. 29.
21. Hrapkiewicz K, Medina L: Clinical laboratory animal medicine, an introduction. 3rd ed. Ames (IA): Blackwell Publishing; 2007. p.133.
22. Hankenson FC, Van Hoosier GL: Biology and diseases of hamsters. In: Fox JG, Anderson LC, Loew FM, et al, editors. Laboratory animal medicine. 2nd ed. New York: Academic Press; 2002. p. 168-197.
23. Harkness JE, Turner PV, VandeWoude S, et al: Harkness and Wagner's biology and medicine of rabbits and rodents. 5th ed. Ames (IA): Wiley-Blackwell; 2010. p. 72.
24. Festing MFW: Hamsters. In: Universities Federation for Laboratory Animal Welfare The UFAW Handbook on the care and management of laboratory animals. 5th ed. New York: Churchill Livingstone; 1976. p. 252.
25. Weir BT: Chinchilla. In: Hafez ESE, editor. Reproduction and breeding techniques for laboratory animals. Philadelphia: Lea and Febiger; 1970. p. 211.
26. Quesenberry KE, Donnelly TM, Mans C: Biology, husbandry and clinical techniques of guinea pigs and chinchillas. In: Quesenberry KE, Carpenter JW, editors. Ferrets, rabbits, and rodents: clinical medicine and surgery. 3rd ed. St. Louis: Elsevier/Saunders; 2012. p. 287-288.
27. Donnelly TM, Quimby FW: Biology and diseases of other rodents. In: Fox JG, Anderson LC, Loew FM, et al, editors. Laboratory animal medicine. 2nd ed. New York: Academic Press; 2002. p. 286.
28. Mans C, Donnelly TM: Disease problems of chinchillas. In: Quesenberry KE, Carpenter JW, editors. Ferrets, rabbits, and rodents: clinical medicine and surgery. 3rd ed. St. Louis: Elsevier/Saunders; 2012. p. 316.
29. Cooper G, Schiller AL: The anatomy of the guinea pig. Cambridge: Harvard University Press; 1975. p. 331.
30. Harkness JE, Turner PV, VandeWoude S, et al: Harkness and Wagner's biology and medicine of rabbits and rodents. 5th ed. Ames (IA): Blackwell Publishing; 2010. p. 52-55.
31. Harkness JE, Murray KA, Wagner JE: Biology and diseases of guinea pigs. In: Fox JG, Anderson LC, Loew FM, et al, editors. Laboratory animal medicine. 2nd ed. New York: Academic Press; 2002. p.209-210.
32. Sisk DB: Physiology. In: Wagner JE, Manning JP, editors. The biology of the guinea pig. New York: Academic Press; 1976. p.83.
33. Harper LV: Behavior. In: Wagner JE, Manning PJ, editors. The biology of the guinea pig. New York: Academic Press; 1976. p. 43.
34. Hawkins MC, Bishop CR: Disease problems of guinea pigs. In: Quesenberry KE, Carpenter JW, editors. Ferrets, rabbits, and rodents: clinical medicine and surgery. 3rd ed. St Louis: Elsevier/Saunders; 2012. p. 302-303.
35. Beregi A, Zorn S, Felkai F: Ultrasonic diagnosis of ovarian cysts in ten guinea pigs. Vet Radiol Ultrasound 1999;40:74-76.

Advanced techniques in rabbit reproduction

M. I. Baracaldo
Baracaldo Veterinary Reproductive Mobile Services, London, ON, Canada

Abstract

The use of assisted reproductive technologies in rabbits, especially the use of artificial insemination (AI) techniques with extended liquid refrigerated semen, is a common practice in European countries such as Italy, France and Spain where there are well-established rabbit meat production industries due to public interest and demand in consuming rabbit meat as part of their daily diets. In the USA and Canada, the rabbit industry is in its infancy and for producers interested in improving their genetic lines, the use and application of AI will be an excellent tool to be considered. This article reviews all the steps involved in the use of AI techniques in rabbits.

Keywords: Artificial insemination, sperm, semen, rabbit, rabbit doe, rabbit buck.

Introduction

The intensive production of rabbit meat is relatively new in comparison with the pork, chicken and beef industries. The industrial production of rabbit meat started in the late 1970s. The 25 countries that are members of the European Union produce 520,000 tons of rabbit meat, a number that represents 50% of the rabbit meat world production. Italy with 215,000 tons, Spain with 120,000 tons and France with 75,000 tons together produce together 80% of the European rabbit meat.[1]

The estimated number of rabbit AIs happening per year in Italy is 5 million; in France 4 million/year and Spain 3.5 million/year are reported (Guy Delhomme, personal communication). For the year 2009, France estimated its rabbit carcass production obtained by AI as 45,800 tons.[2]

In Canada and the USA, rabbit meat production is in its infancy and the consumption of rabbit meat is extremely low. In Canada, the consumption of rabbit meat between 2008 and 2011 fluctuated between 18.7 and 22 grams per capita.[3]

The market for rabbit meat in the USA is small. However, rabbit meat is lower in fat, cholesterol, and calories, and higher in protein than beef, chicken, turkey, or pork, and these ideal nutritional attributes hopefully will increase demand. Rabbit production in the USA grew from being predominantly for home consumption to a large-scale commercial operation of about 200,000 rabbit producers in 2004. Researchers estimate that about 8 million rabbits are produced each year and that between 8 and 10 million pounds of meat are consumed annually.[4] Hopefully this healthier source of animal protein will continue to attract more American and Canadian people to consume rabbit meat, and a real growth and development of the rabbit meat industry can become a reality similar to what happened in Italy, Spain and France.

This article is a summary of the basic steps involved in use of AI as an assisted reproductive technology in the rabbit industry.

Reproductive anatomy of the rabbit doe

The rabbit doe reproductive system has the following organs: two ovaries, two oviducts and two uterine horns. These two uterine horns are separated from the vagina by the presence of two uterine cervices. Each rabbit doe ovary is approximately 1 cm wide by 2 cm long. On each ovary follicles are observed in different stages of maturation and during pregnancy it is possible to see the corpora lutea that secrete progesterone. The weight of each ovary varies depending on their physiological stage. The oviducts are tubular sinusoidal structures that can be 10-16 cm long and have the following three components: 1) the infundibulum that partially covers the ovary and functions to receive the ovum at the moment of ovulation; 2) the ampulla where the fecundation process happens and which is internally covered by ciliated cells that facilitate gametes transit of gametes; and 3) the isthmus which is thin and covered by mucus, secretory cells, and a higher number of ciliated cells. The uterus has two uterine horns where the gestation process occurs. Each horn is cylindrical, measures 10-12 cm long and has three

circumvolutions. The uterine horns will receive the embryos that will become implanted in the endometrium. The two horns are connected independently to the common vagina through two individual uterine cervices of approximately 2 cm long each. The presence of bicornual cervices in rabbits does not allow embryo migration to occur from one horn to the other. The vagina is 4-8 cm long. It is possible to see the urethral orifice caudally. The vagina continues caudally with the vestibule that measures approximately 2-3 cm. The vestibule ends at the external vulva. The vulvar lips change color depending on the sexual receptivity of the rabbit doe. The clitoris is well-developed and is found on the ventral commissure of the vulva.[5]

Follicular growth in rabbit does

Oogenesis is completed in rabbit does during the first two weeks of life with simultaneous growth of primordial follicles. The ovaries of four to eight week-old rabbit does already show follicles at early stages of development. It is well-known that rabbit follicles can produce polyovular structures containing two to three oocytes that can develop depending on their intrafollicular position. Follicular peripheral oocytes can have less opportunity of resuming meiosis when compared to centrally localized oocytes. For this reason, not all the oocytes present inside one follicle can be fertilized.[6]

Age at puberty in rabbit does depends on the breed, nutrition and management conditions. Rabbit does of small breeds can reach puberty at 3-4 months, medium breeds at 4-5 months, and large breeds at 8-9 months of age.[7]

It is recommended to breed primiparous rabbit does when they have reached 80% of their adult body weight.[8] Table 1 shows the recommended age and a weight for primiparous rabbit does, according to breed size:

Table 1. Recommended age and weight for primiparous rabbit does according to breed size. [8]

Breed	First breeding age (weeks)	Weight (kg)
Small breeds	20	2.8
Medium breeds	23	3.6
Large breeds	27	4.8

After puberty, rabbit does do not have a well-defined estrous cycle. They grow waves of follicles that continuously develop to the antral stage under the influence of follicle stimulating hormone (FSH). The presence of large antral ovarian follicles that increase plasma estrogen concentration will initiate sexual receptivity in rabbit does that lasts for several days (erroneously considered to be permanently in estrus).[6] During this period of receptive behavior, rabbit does can be mounted by the rabbit buck and become pregnant.[7] The follicular waves will regress at approximately 7-10 day intervals.[6]

In receptive multiparous rabbit does, their fertility is higher when the vulva color is pink or red; this color change is associated with elevated estrogen concentration. Fertility rates are improved by up to 10% if rabbit does are inseminated when the vulva is red, turgescent pink or turgescent purple. The number of rabbit does that kindled following AI was significantly higher when the vulva color was red (55.2%) or pink (51.5%) than when the vulva color was white (33.3%). However, failure to conceive in does with pale or white vulva has been observed even after natural mating.[9]

It is well known today that rabbit does that are showing receptive behavior at the time of AI will determine the fertility, ovulation frequency, fertilization rate and prolificacy results, facts that will result from the ovulation rate, embryo and fetal survival. For instance, the productivity of primiparous receptive does (6.3 weaned rabbits/AI) is higher than in non-receptive does (1.6 weaned rabbits/AI). The same is observed in multiparous receptive and non-receptive does: 7.8 vs. 2.9 rabbits/AI respectively.[6]

Induced ovulators

Rabbit does are induced ovulators, as was discovered in 1905 by Heape. Ovulation is induced by mating with the rabbit buck and happens 10-12 hours post-mating. The neuro-hormonal response of induction of ovulation in rabbits has two pathways:[10]

- A neural pathway that after mating activates several sensory areas whose evoked signals are transmitted via neural afferent pathways along the spinal cord, in the brainstem and hypothalamus.[6]
- A hormonal pathway, that sends the signal from the central nervous system to the ovum by producing the ovulation per se.[10] The hormonal mechanism after copulation is the release of gonadotropin releasing hormone (GnRH) from the hypothalamus that induces the release of luteinizing hormone (LH) from the pituitary gland, with respective ovulation of follicles.[5] It has been demonstrated from direct sampling of portal blood from the pituitary stalk of rabbits that GnRH increases rapidly after coital stimulation, with peak secretion within 1-2 hours. Plasma LH levels start to rise within 3 min after mating and reach a plateau within 15 to 75 min.[11] The LH surge preceded by the GnRH rise has a maximal release 60-90 minutes after mating and gradually decreases within the next 4-6 hours post-mating.[6]

Fertilization, gestation and pseudopregnancy

The success of fertilization requires coordination between the time of transportation and duration of viability of the gametes. Ovulation will occur 10-12 hours after mating. Ova will be fertilized 2-6 hours after ovulation or 12-18 hours after mating. Spermatozoa should reach the fertilization location before ova arrive, and during this waiting time each spermatozoon should undergo a series of modifications (sperm capacitation) that will allow penetration of the ovum for fertilization. The duration of pregnancy in rabbit does is 30-31 days, with a range of 28 to 35 days.[5]

If for any reason the released ova are not fertilized after ovulation, the rabbit doe will begin a stage of pseudopregnancy that lasts between 15 and 18 days with development of corpora lutea and uterine changes similar to those of a pregnant animal. During any pseudopregnant period the rabbit doe is not fertile. Pseudopregnant animals can be identified because towards the end of the stage (around 16 days after mating) the level of progesterone decreases due to the involution of the corpora lutea, triggering the development of maternal and nesting behaviors. In pregnant does, the maternal and nesting behaviors begins after 25 days of pregnancy.[7,8]

Rabbit bucks

Mature rabbit bucks are fertile during the whole year, although they may show decreased fertility during the summer due to the variation in photoperiod and increase in environmental heat.[8]

The majority of rabbit bucks will try mating a rabbit doe a few seconds after she is introduced into the cage where the buck regularly lives. Natural mounts will happen very quickly with intense pelvic thrusting by the buck. Natural breeding lasts approximately 70 seconds, ranging from 5 to 300 seconds and may be repeated several times.[8]

Although the first spermatozoa could be observed around 110 days of age in rabbit buck ejaculates, bucks should be used for the first time for reproductive purposes when they reach five months of age by introducing them progressively from one breeding per week to a maximum of six to eight breedings per week by the time they reach eight to ten months of age. At this age, breeding can be done every second day.[8]

In ideal conditions for AI purposes, the room where the rabbit bucks are living must have controlled lighting, temperature, humidity, and ventilation. The photoperiod should be of 16 hours of daylight. The ideal room temperature for rabbit bucks should be at 21°C. The ideal range of controlled relative humidity must be between 50-60%. Air ventilation should be between 20-40 cm/second.[1]

Rabbit bucks that are two to five months old should be placed in replacement cages. When they reach 4.5 months of age, they should have an exhaustive physical evaluation and those who pass it should

be moved to production cages. Between five to seven months of age, the rabbit bucks should be trained to be collected and to ejaculate into an artificial vagina (AV). This period of time will also be used to evaluate the semen quality of the bucks. Approximately 10-30% of rabbit bucks are rejected and will not be used for AI purposes due to lack of adaptation to the AV or poor production and low quality of semen.[1]

Preparation of semen doses for artificial insemination

Semen is a mixture of spermatozoa, produced by the two testicles, and seminal plasma secreted at different sites by the accessories glands and by the epididymides; these are combined at the time of ejaculation. Seminal plasma can also contain seminal granules other particles of different size produced by the accessory glands which can affect the spermatozoa behavior during the transit along the rabbit doe reproductive tract.[12]

Rabbit buck semen collection

In the rabbit industry, ejaculates should be collected with the most hygienic conditions and in the most efficient way. For this reason it is ideal to have at least the same number of AVs and collection tubes than the number of ejaculates needed per day. If each ejaculate is collected with a different AV, it will prevent any potential contact between animals, promoting optimal sanitary conditions. It is also important to send samples to an external laboratory for periodic microbiological culture and evaluation of ejaculates according to veterinary instructions.[1]

The most popular method of semen collection from rabbit bucks is use of an AV. The most popular AV model has a semi-rigid outer case and an internal liner that can be made of rubber or latex (Figure 1). The bottom of the semi-rigid case has two openings of different sizes. The largest opening is to place the ejaculate collection tube and the smaller opening is to fill the AV with hot water.[1] The ideal AV temperature for rabbit buck semen collection is 45-50°C.[7,13] In order to reach this temperature range, the AVs are filled with hot water and kept inside an incubator before they are used for the collection.[1,7,13] If the AV temperature is too hot, the buck could contaminate the ejaculate with urine or could burn the penile mucosa producing balanitis, or the high temperature could cause damage to the spermatozoa collected.[1,7] Rabbit bucks respond to appropriate stimulation created by the AV pressure and temperature so it is important to make sure the water pressure and temperature between the outer case and the rubber liner are appropriate.[1,7,13] If the temperature is too cold (below 40°C), the buck will refuse to jump.[1,7]

Semen collection normally happens inside the rabbit buck's cage. For this purpose a decoy animal (rabbit buck or doe),[1,7,13] or the arm of the semen collector covered with rabbit skin that simulates the decoy rabbit are used (author's personal experience). When a decoy animal is placed inside the cage, the rabbit buck will get closer and try to jump it. At this moment the hand holding the AV has to be directed to the rabbit buck's penis to perform the collection.[7] When using the arm covered with rabbit skin, the buck will jump into the arm and start thrusting. At this moment the AV has to be directed to the rabbit buck's penis to perform the collection (author's personal experience; Figure 2).

In the rabbit AI industry, the recommended collection frequency is two ejaculates per rabbit buck per week with a time interval of 15-20 minutes between collections.[1,13] In high demand bucks, it is possible to perform up to four extractions per week divided into two days per week and two collections per day.[1]

The collection tubes containing the ejaculates should enter the laboratory through a pass-through window located between the rabbit buck's room and the laboratory.[1] A pass-through window contributes to clearly separate the dusty and non-sterile environment where the bucks are collected from a clean and disinfected laboratory environment where the samples should be evaluated and processed making a great contribution to biosecurity, especially when processed extended semen samples will be shipped to other farms to inseminate other animals (author's personal experience).

Rabbit buck semen evaluation

When using AI, an ejaculate from one rabbit buck can be used to inseminate a large number of rabbit does. In the rabbit industry, a single ejaculate can be divided into 20-50 doses for insemination of rabbit does.[14]

Variation in the seminal characteristics of rabbit bucks is known to be affected by many factors such as genetic strain, feeding, health status, rearing condition, season, age and collection frequency. Substantial differences among laboratories may increase variability in the evaluation of sperm parameters (sperm counts, motility and morphology).[12]

After the collection tubes containing the ejaculates enter the laboratory, they should be placed in a water bath at 37°C in order to prevent thermal shock.[1]

Semen macroscopic evaluation

A visual observation is done in order to determine the semen color, odor and volume.[1]

Appearance. The ejaculate could contain a mucus plug, urine, calcium carbonate crystals or blood.[1] If a mucus plug that is produced by the accessory sex glands is present, it should be removed because it causes sperm agglutination.[7]

Color. Evaluation of color helps to determine the ejaculate density. Different color spectra that can be observed in ejaculates include: ivory (ideal), white milky, semi-transparent white, or cream.

Odor. It should not be unpleasant.

Volume. It should be measured in the graduated tube after removing the mucus plug.[1]

Semen dilution

After visual evaluation, the ejaculate should be diluted 1:5 by adding warm extender to the semen collection tube. There are different commercial semen extenders available in the market. Liquid, powder and gel extenders are available. Liquid and gel extenders should be kept refrigerated while powder extenders should stay in a cool place and away from direct light. It is important to make sure that the semen extender is prepared, warmed and kept at 37°C before the semen evaluation has started.[1] Examples of commercial rabbit semen liquid extenders are Cudil™ manufactured by Magapor from Spain[1] and Galap™ manufactured by IMV Technologies from France.[15]

Microscopic evaluation

The ejaculates of rabbit bucks contain seminal granules. These particles are secreted by the prostate gland, mainly in the first lobe, called pro-prostate. These semen granules are not homogeneous and are composed by different populations of vesicles. They are of different sizes (0.5-6 mm diameter; Figure 3) and are generally surrounded by a bilaminar membrane containing a scarcely organized electron dense material. Researchers have suggested that these semen particles modulate the capacitation process and acrosome reaction of spermatozoa, their kinetics, the immune-response of female tracts, and the transit of spermatozoa in the female tract. In the rabbit species these granules are mainly involved in the control of capacitation and the acrosome reaction. It has been shown that the presence of seminal granules significantly reduces the response of spermatozoa to *in vitro* inducers of the acrosome reaction and as a result the level of capacitated spermatozoa is almost equal to zero. In contrast, when granules are removed by using Percoll® centrifugation the decapacitative effect is virtually eliminated. As commented earlier, ovulation in rabbit does occurs about 10-16 hours after mating and during this lag-phase rabbit spermatozoa must avoid premature capacitation and acrosome reaction, and the seminal particles contribute to delaying this process.[12]

Semen should be evaluated with a microscope that has a 37°C warming stage, and a warming plate that will also be used to keep the microscope slides and cover slips warm at 37°C.[1]

The following are the microscopic characteristics to be evaluated:

1. Individual motility observing the progressive motility of spermatozoa. This evaluation can be done with a phase-contrast microscope and a magnification between 200X to 400X (author's personal experience). The individual progressive motility can be done as a subjective evaluation (Table 2) or as an objective evaluation with a Computer Assisted Sperm Analysis system.[1] It is necessary to have ejaculates

with individual progressive motility equal to or greater than 70% in order to be included in pooled extended semen doses.[23]

Table 2. Subjective mark scale and equivalency in progressive motility

Subjective Mark scale	Progressive motility (%)
0	0
0.5	1-10%
1	11-20%
1.5	21-30%
2	31-40%
2.5	41-50%
3	51-60%
3.5	61-70%
4	71-80%
4.5	81-90%
5	91-100%

2. Sperm concentration. After performing a dilution of the sample, the sperm concentration can be calculated with help of counting systems such as the Newbauer chamber, the Bürker chamber,[1,7] or the Nucleo Counter SP100.[1]

3. Sperm morphology. The percentage of normal spermatozoa can be evaluated by placing a drop of semen on a glass slide and staining it with eosin-nigrosin. An eosin-nigrosin stained semen smear is made, allowed to dry and observed under the microscope at 1000X magnification[7] with oil immersion (author's personal experience). An ejaculate will be accepted if it has more than 70% morphologically normal spermatozoa.[7]

4. Other semen microscopic characteristics. Depending on the laboratory, additional characteristics can be evaluated and scored such as the presence of sperm agglutination, presence of seminal granules, the presence of dead spermatozoa, cytoplasmic droplets and others.[1]

The most common semen parameters found in rabbit bucks for AI purposes are:

Ejaculate volume: 0.3 to 0.8 ml.[8] On some occasions, volumes of 3 to 5 ml could be collected.[7]

Spermatozoa concentration: 150-300 million/ml with a range of 50 to 500 million spermatozoa per ml of semen.[8]

Some laboratories give a score to each semen characteristic evaluated in the ejaculates collected. The ejaculates that have a final passing score will be added to the semen pool that will be used to prepare the insemination doses.[1]

Definitive dilution and storage

The most accepted volume to inseminate a rabbit doe is 0.5 ml of extended liquid semen that should be stored at 18°C. When planning to use extended fresh semen doses within the next 12 hours, each 0.5 ml dose must have 6 to 8 million spermatozoa.[23] When planning to use extended fresh semen doses 12 to 36 hours after collection, each semen dose should have a minimum of 12-16 million spermatozoa.[23] Some companies use as a final target 15 million spermatozoa per insemination dose.[1]

When the volume and concentration of an extended semen pool is known, it is possible to dilute the concentration to 30 million spermatozoa per ml of extended semen.[1]

Calculation to prepare pooled semen doses:

- Pool volume (ml) X Pool concentration = Final Volume X Target concentration of $30X10^6$ (spz/ml)
- Final Volume = Pool volume (ml) X Pool concentration/$30X10^6$ (spz/ml)

- The final extender volume that is needed will be the result of subtracting the final volume from the pool volume.
- Final volume X 2= Number of doses produced

Calculation example: Pool volume is 250 ml with a final concentration of $60x10^6$ (spz/ml). The target final concentration is $30X10^6$ (spz/ml)
Final Volume = $(250 \times 60x10^6)/ (30X10^6)$ = 500ml
Extender volume to be added: 500 – 250 = 250 ml
Total number of doses produced: 500 ml X 2= 1,000 doses

The extender will be slowly added to the pool and must have the same temperature as the pooled semen. Once the pool has been fully diluted to the final concentration, a sample should be taken to evaluate the individual motility of the cells present in the pool. After the final dilution, the pooled semen should be refrigerated at 18°C until use.[1]

Methods to synchronize estrus and sexual receptivity in rabbit does

The extensive and wide application of AI in European rabbit farms has evolved to current use of methods of production such as "cycled production" in which all the does in a batch must be inseminated on the same day. Researchers found a strong antagonism between lactation and reproductive functions in non-receptive rabbit does because at the moment of AI these lactating and non-receptive does had poor reproductive performance. This difficulty was a serious problem in intensive rabbit production because does had to be inseminated during the first days of lactation, from 0 to 11 days post-partum. It is important to clarify that with natural mating this situation would not be happening because in general non-receptive does refuse to mate. As was explained earlier, sexual receptive behavior in does is correlated with the presence of more pre-ovulatory follicles on the rabbit ovary and higher concentration of circulating estradiol. High, regular and intensive production levels require the use of techniques to induce and synchronize estrus and receptivity in lactating does.[16]

A very common system of production in commercial rabbit farms is to use batch management systems that will create groups of animals (batches) that are in the same physiological stage of production such as insemination, kindling, weaning, etc. One of the most common production systems is the 42-day batch management system that requires the rabbit producer to perform AI 11 days after kindling. In the 42-day batch management system the does' reproduction rhythm is based on the time interval that happens between two inseminations performed on the same animal. After the first insemination there are 31 days of gestation, then parturition, and the second AI is done 11 days later in the same lactating post-partum doe, for a total of 42 days between inseminations.[17]

In order to synchronize estrus and induce receptive behavior in lactating does 11 days post-partum, it is very common to use a hormonal treatment 48 hours before AI that consists of g 20 IU equine chorionic gonadotropin (eCG) intramuscularly.[18] The same hormonal protocol can be used in primiparous does.[17]

Additional biostimulation programs have been developed as alternatives to stimulate rabbit does for AI programs. Practical examples of biostimulation are increased lighting from 8 to 16 hours per day in does prior to insemination, separation of the lactating mother from the litter for 24-48 hours before AI, and nutritional flushing.[17,19]

Artificial insemination

There are several restraining techniques that can be used in order to perform AI in rabbit does:

- Insemination with rabbit doe in vertical position. The animal is restrained by the cervical area with one hand and by the inguinal zone with the other hand, as if performing an abdominal palpation. This technique requires two people.[1]
- Insemination with rabbit doe in inverted position. The main advantage of this restraint technique is that the doe does not adopt a defensive position because the insemination will be done quickly. This technique requires two people.[1]

- Cannon restraining device. In 1990, a system was created in Italy that permits only one operator to perform AI. The system is basically a cylindrical restraining device that is 24 cm long and has an internal diameter of 15 cm. The system has a 23 degree slope and the bottom part is 12 cm longer than the top area in order to hold the rabbit's abdomen or rump, leaving the rump exposed for AI. The inseminator can work hands-free and inseminate 80-100 does/hour without excessive doe manipulation.[7]

Rabbit does that will be inseminated should be in excellent health and be sexually receptive, which can be confirmed by observing the red color of the vulvar lips.[1]

There are different instruments that can be used to perform AI in rabbits:

- A pipette made of plastic or glass that is approximately 16 cm long and has an external diameter of 6 to 7 mm. This pipette is slightly curved on the tip, approximately 15 degrees. The other end should be connected to a syringe in order to allow the aspiration of the semen dose into the pipette (Figure 4).[7] The pipette should be carefully introduced with the curvature directed towards the dorsal area of the vagina. During intromission the pipette will rotate spontaneously and penetration will become easier. The pipette should be introduced inside the vagina as far as possible. As soon as it stops, the pipette should be slightly withdrawn and the syringe plunger pushed to inject the semen (Figure 5). The pipette should enter an average of 15 cm inside the rabbit doe, depending on the age and size of the animal. In order to remove the pipette, it has to be pulled slowly. Rotation will occur and it will come out without any difficulties. After the pipette has been removed, it is important to look for the presence of pus or blood on the pipette tip, elements that will suggest infection or laceration.
- 0.5 ml semen straws can also be filled with liquid extended semen. The straw should be placed inside a metallic AI gun. A specific plastic sheath that perfectly fits the tip of the semen straw will then be used to cover the metallic insemination gun (Figure 6; author's personal experience). The insemination technique should be done using the same steps explained with the plastic/glass pipette.

Induction of ovulation after artificial insemination

In order to induce the ovulation of follicles after AI is performed, it is necessary to administer GnRH (20 μg/animal intramuscularly). In an effort to induce ovulation afterinsemination, researchers have added GnRH analogs such as ethylamide to the semen dose (25 μg/semen dose intravaginally) which resulted in similar fertility and prolificacy and provides an alternative to the intramuscular administration of GnRH or its analogs.[20] Similarly, another research group added the synthetic GnRH analogs buserelin (10 μg/ml of semen extender) or triptorelin (10 μg/ml of semen extender) in order to be absorbed by the vaginal mucosa, and obtained acceptable fertility and prolificacy.[21] A different method that has also been investigated is the use of vasectomized bucksAI, which produces results similar to natural breeding. This technique has the disadvantage of increased time to perform the procedure, plus the necessity of having a group of vasectomized males on the farmand for these reasons this method is not very popular.[18]

Pregnancy diagnosis

Pregnancy cn be diagnosed by transabdominal ultrasonography as early as seven days after ovulation when early embryonic vesicles can be observed when using the appropriate transducer and unit equipment.[22]

Producers working in batching management systems could organize the schedule to perform pregnacy diagnosis by transabdominal palpation or ultrasonography at 14 days post-ovulation.[17]

References

1. Magapor: Inseminación Artificial en Cunicultura. Zaragoza (Spain). Available from: http://www.magapor.com/images/Ayuda/4.pdf.

2. Lebas F: Situation cunicole en France en 2009: Performances moyennes des élevages selon les résultats de RENACEB pour l'année 2009, situation du marché cunicole français et premières évaluations pour l'année 2010. Cuniculture 2010;37:74-82.
3. Agriculture and Agri-Food Canada: Consumption per capita, rabbit supply-Canada 2011. Available from: http://www.agr.gc.ca/redmeat/rpt/11tbl38a_eng.htm#t2.
4. Beal MN, McLean-Meyinsse PE, Atkinson C: An analysis of household consumption of rabbit meat in the southern united states. J Food Distr Res 2004;35:24-29.
5. Martín M: Reproducción: I.-Anatomía & fisiología. Cogal, 2003 May; Sect. Cuadernos Prácticos- Reproducción en la coneja. p. 27-34.
6. Boiti C, Besenfelder U, Brecchia G, et al: Reproductive physiopathology of the rabbit doe. In: Maertens L, Coudert P, editors. Recent advances in rabbit sciences. Melle: ILVO; 2006. p. 3-19.
7. Zarate-Báez PI: La reproducción del conejo doméstico (oryctolagus cuniculus) y el desarrollo de las nuevas tecnologías reproductivas (dissertation). Morelia (Michoacán): Universidad Michoacana de San Nicolás De Hidalgo; 2006.
8. Gonzalez-Redondo P: Taller de Cunicultura – Asignatura Producciones de Aves y Conejos (course notes). Universidad De Sevilla- Escuela Universitaria De Ingeniería Técnica Agrícola - Departamento De Ciencias Agroforestales -Área De Producción Animal. 2007; p. 1-51. Available from: http://centro.us.es/gprodanim/PCA/Tallercunicultura.pdf.
9. El Gaafary MN, Marai IFM: Artificial Insemination in rabbits. In: Baselga M, Marai I FM, editors. Rabbit production in hot climates. Zaragoza: CIHEAM; 1994. p. 95-107 (Cahiers Options Méditerranéennes; n. 8).
10. Rodriguez-Serrano TM: Inducción de la ovulación. Boletín de Cunicultura 2004;134:51-54.
11. Rebollar PG, Dal Bosco A, Millán P, et al: Ovulating induction methods in rabbit does: the pituitary and ovarian responses. Theriogenology 2012;77:292-298.
12. Castellini C: Semen production and management of rabbit bucks. Proc 9th World Rabbit Cong; 2008. p. 265-277.
13. Kitajima S: Improvement of rabbit production. In: Houdebine LM, Fan J, editors. Rabbit biotechnology: rabbit genomics, transgenesis, cloning and models. New York: Springer; 2009. p. 3-7.
14. Lavara R, Moce´ E, Lavara F, et al: Do parameters of seminal quality correlate with the results of on-farm inseminations in rabbits? Theriogenology 2005;64:1130-1141.
15. IMV Technologies: Available from: http://www.imv-technologies.com/our-solutions/rabbit/detail/product/galap-dilueur-pret-a-lemploi-pour-semence-lapin.html.
16. Theau-Clément M, Boiti C, Bonanno A, et al: Alternative methods for the synchronization of oestrous in lactating rabbit does. Proc 9th World Rabbit Cong; 2008. p. 21-37.
17. Martín M, Ferreres J: De monta natural a Inseminación Artificial. Boletín de Cunicultura, 2003;127: 6-13.
18. Quintela LA, Vega MD, Becerra JJ, et al: Inducción de la ovulación en conejas sometidas a inseminación artificial mediante la adición de buserelina a la dosis seminal. Cogal, 2003 May; Sect. Investigación- Inducción de la ovulación. p. 5-11.
19. Gerencsér Z, Matics Z, Nagy I, et al: Effect of a light stimulation on the reproductive performance of rabbit does. Proc 9th World Rabbit Cong; 2008. p. 371-374.
20. Quintela LA, Peña AI, Vega MD, et al: Inducción de la ovulación en conejas mediante la administración intravaginal de (des-Gly10, D-Ala6)-LHRH ethylamide: Estudio preliminar. Proc II Cong Ibérico de Cunicultura; 2007. p. 37-39.
21. Viudes de Castro MP, Lavara R, Marco Jiménez F, et al: Ovulation induced by mucosa vaginal absorption of buserelin and triptorelin in rabbit. Theriogenology 2007;68:1031-1036.
22. Gutierrez HE, Zamora FMM: Ultrasonoghraphy study of rabbit pregnancy. Proc 8th World Rabbit Cong; 2004. p. 276-280.
23. Lavara R, Mocé E, Vicente JS: Buenas prácticas en inseminación atificial I: Preparación de dosis seminales. Los factores responsables de la variación de los resultados de fertilidad y prolificidad de la IA. Boletín de Cunicultura 2003;127:14-23.

Figure 1. Left: Rabbit AV; semi-rigid body with the rubber inner liner already in place . Right: Assembled rabbit AV showing the semen collection tube and a protective case to cover the ejaculate during collection. (Pictures courtesy of Guy Delhomme, IMV Technologies, France).

Figure 2. Left: Arm covered with rabbit skin and holding an AV ready for semen collection from a rabbit buck. Right: Rabbit buck semen collection. The rabbit buck has jumped the rabbit skin covered arm and is already thrusting inside the AV held by the collector (Author's personal pictures).

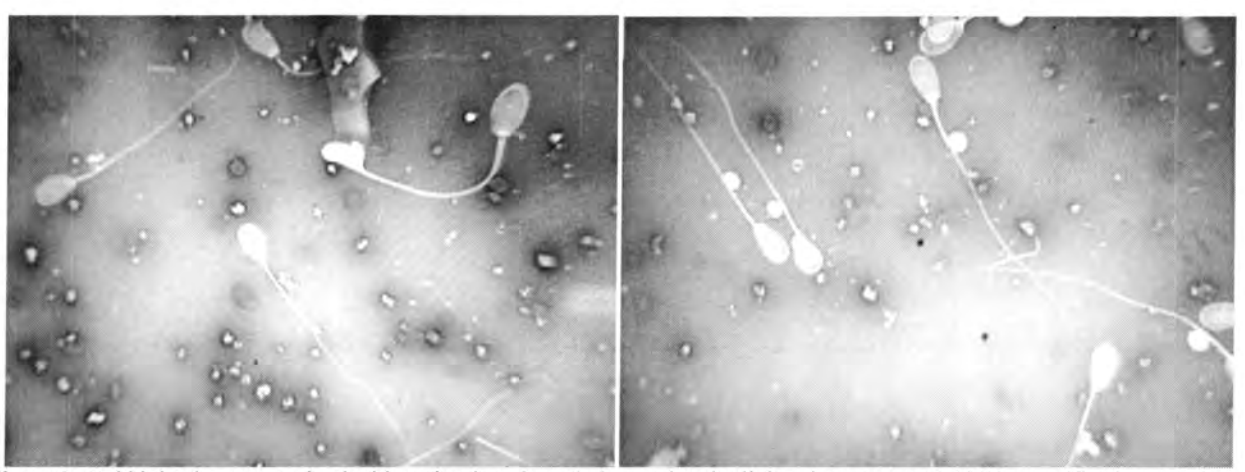


Figure 3. Rabbit buck semen stained with eosin-nigrosin and observed under light microscopy at 1,000X magnification. Notice the presence of seminal granules of different sizes around the white spermatozoa. (Author's personal pictures).

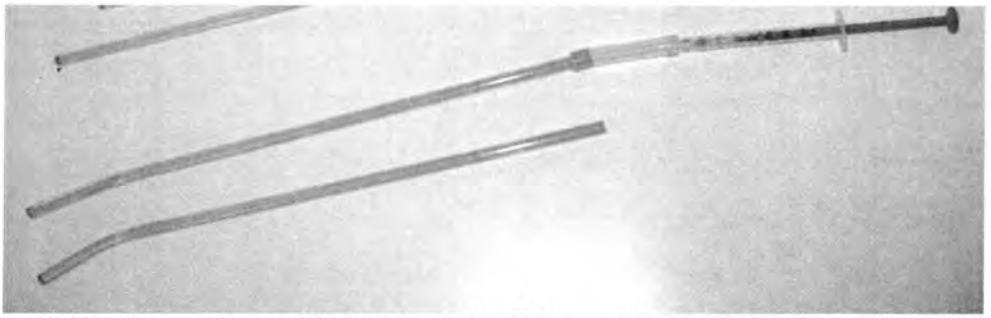


Figure 4. Disposable blue pipette with curved tip for doing AI in rabbit does. It can be connected to a syringe (Picture courtesy of Guy Delhomme, IMV Technologies, France).

Figure 5. Artificial insemination of a rabbit doe that is being restrained by an operator. The inseminator is using the disposable blue pipette to perform the insemination and injecting the semen with a syringe. (Picture courtesy of Guy Delhomme, IMV Technologies, France).

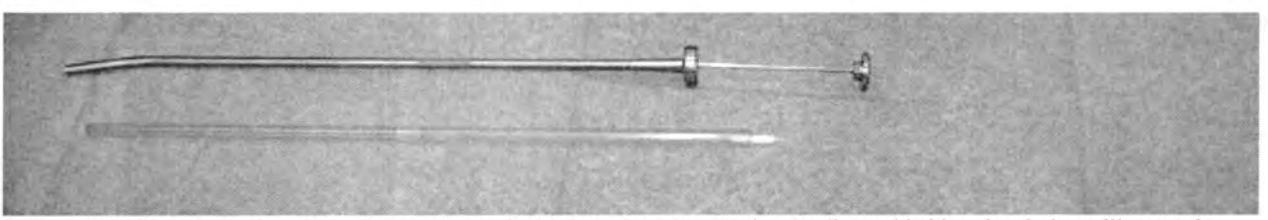


Figure 6. Metallic AI gun for rabbits that can be used with 0.5 ml straws. Notice the disposable blue sheath that will cover the metallic gun and fits perfectly well with the straw tip. (Picture courtesy of Guy Delhomme, IMV Technologies, France).

(Editor's note: The photographs in this paper are available in color in the online edition of Clinical Theriogenology.)

Ovariectomy vs ovariohysterectomy: should the uterus stay or should it go?

Roberto E. Novo
Columbia River Veterinary Specialists, Vancouver, WA

Objectives

- To discuss the pros and cons for ovariohysterectomy (OVH) and ovariectomy (OVE) procedures.
- To review the short-term and long-term complications associated with each procedure.
- To provide an evidence-based approach for making the decision of performing either an OVE or an OVH.

Key points

- From a technical perspective, the OVE is less invasive and less time consuming than the OVH procedure.
- Complications associated with the OVE procedure would be similar to those associated with the ovariectomy portion of the OVH procedure. However other complications associated with removal of the uterus in an OVH would not be expected with an OVE.
- The uterus should always be removed if there is evidence of uterine pathology.
- Complete removal of the ovaries prevents the occurrence of pyometra. In order to develop cystic endometrial hyperplasia-pyometra complex, progesterone must be present either from the ovary at the luteal phase of the estrous cycle or from an exogenous source. Post-gonadectomy endogenous progesterone occurs from ovarian remnant syndrome.

Overview

There has been a long lasting argument regarding whether or not the uterus should be removed during gonadectomy in female patients. In the USA, the preferred method is performing OVH, whereas in most European countries, the OVE is the preferred method. In the USA, there has been a recent greater push to switch to the OVE procedure; however, much resistance is still present. So why is that? With a quick search of the Veterinary Information Network, you can quickly determine the strong feelings present for one procedure versus the other. Some are founded on experience, and some are founded on misinformation. In most veterinary educational programs in the USA, the OVH procedure is taught primarily. Most recently, with the increased use of minimally invasive surgical techniques, laparoscopic OVE has gained popularity, bringing up the discussion once again.

At this time there are few randomized studies comparing complications after OVE and OVH. Based on the evidence that is currently available in the veterinary literature, there is no support for any benefit and therefore no indication for removing the uterus during routine neutering of healthy bitches.

Surgical technique

From a technical perspective, OVE is an easier, less invasive surgery when compared to OVH. Both require a median celiotomy, however for the OVE the incision is smaller and located more cranial, at the level of the umbilicus. This allows for better exposure of both ovaries. In the OVH, the incision is extended caudally towards the pubis, to allow ligation of the uterine body cranial to the cervix. The identification and exposure of the ovary is performed similarly for both procedures. However, due to the OVH requiring a longer incision caudally, most veterinarians do not extend the incision cranially enough to allow for adequate exposure of both ovaries. Presumably this is done because the ovary can be exteriorized after the suspensory ligament is broken, whereas the uterus is more fixed. As well, most surgeons want to make a small approach for the procedure, expediting closure and decreasing postoperative pain. The more caudal incision does make access to the right ovary, which is positioned more cranially, more challenging. Although it is possible to perform an OVH through a small incision, atraumatic technique and correct placement of the uterine ligatures near the cervix requires a longer

celiotomy. In the OVE, because the celiotomy is shorter and because the broad ligaments and uterus are not disrupted, there should be less surgical trauma.

In one study from Utrecht University (Peeters, 2011), there was no significant difference in the total surgical time, pain scores, and wound scores between OVE and OVH. In this study, all surgeries were performed by a board certified surgeon. There may be some differences between a new graduate performing these procedures. However, with time and experience, the time difference should diminish.

Postoperative pain

Pain following either OVE or OVH was evaluated in a prospective clinical trial (Peeters, 2011). There was no significant difference in Glasgow composite measure pain scale between groups, and rescue analgesia was not required for any dog. All dogs in this study received carprofen prior to and following surgery, as well as buprenorphine for the first 24 hours postoperatively. With either procedure, the use of good preemptive and postoperative analgesia should minimize any difference between surgical procedures.

Postoperative complications

The primary rationale for selection either OVE or OVH is often related to the frequency of short-term and long-term complications. In a retrospective study of 62 dogs that had OVH, 17.7% developed complications. Most complications are minimal, consisting of incisional inflammation and gastrointestinal upset, however more severe complications such as intra-abdominal hemorrhage, vaginal bleeding, ureter ligation, granuloma formation, and ovarian remnant syndrome can occur with high frequency.

Short term complications

Intra-abdominal hemorrhage. Life threatening hemorrhage can develop with either procedure and may occur from the ovarian or uterine pedicles, and the broad ligaments. In OVH surgery, the frequency of hemorrhage is greater in dogs weighing >22.7 kg and in procedures performed by inexperienced surgeons. The frequency of hemorrhage is reported between 6.4% and 20% of dogs. Bleeding was most often reported from the right ovarian pedicle. Hemorrhage was determined to be the most common cause of death after OVH in large breed dogs. Unfortunately the incidence of hemorrhage following OVE has not been reported. However, Peeters (2011) reported no significant difference in surgical blood loss between both procedures during surgery, when performed by an experienced surgeon. In this study the suspensory ligament was also coagulated prior to transection. In another study evaluating OVH at a teaching institution (Berzon, 1979), 9% of dogs developed intraoperative or postoperative hemorrhage. Thus, comparing OVE and OVH, one can presume that the likelihood of hemorrhage from the ovarian pedicle should be similar. In theory, there is additional risk with the OVH of bleeding from the broad ligament and uterine vasculature. Surgical experience and surgical approach are significant factors impacting risk of intra-abdominal hemorrhage.

Vaginal bleeding. Vaginal bleeding has been reported to occur in up to 15% of patients undergoing OVH. Causes for vaginal bleeding include the placement of transfixation ligatures and erosion of the uterine vessels from non-absorbable multifilament ligatures. This complication would not be expected in OVE patients. However, transaction of the uterine horn during OVE could cause vaginal bleeding, so care should be taken during ligation of the uterine horn tip vasculature and during transection at the proper ligament.

Ligation of the ureter. Although ligation of the ureters during either procedure is uncommon, the complications can be severe. Ligation can occur proximally during ligation of the ovarian pedicle if the ligature is placed deep at the base of the abdominal wall. If exposure is inadequate the caudal pole of the kidney may not be visible and the proximal ureter can be incorporated. More commonly, ligation of the distal ureter occurs during placement of the uterine body ligature. This occurs more commonly if the

urinary bladder is distended and the trigone is therefore displaced more cranially. One report from the University of Utrecht reported direct ligation of the ureter at the ovarian pedicle in 11% of dogs and ligation of the distal ureter at the uterine ligature in 17% of dogs. Therefore the chances of ligation of the ureter should be similar at the ovarian pedicle between both procedures. The risk of distal ureter ligation is only a factor for the OVH.

Ovarian remnant syndrome. Ovarian remnant syndrome is a disorder characterized by the development of functional residual ovarian tissue after OVH or OVE. Recurrent estrus and development of pyometra can be seen in these patients. This occurs due to incomplete removal of the ovary usually during transaction of the pedicle. Any small remaining remnant of ovarian tissue can re-vascularize and become functional again. There currently are no reports of ectopic ovarian tissue in dogs, therefore this syndrome is considered to be directly related to remaining functional ovarian tissue after surgery.

Recurrence of estrus following OVH has been reported in 17-43% of dogs. Ovarian remnants appeared to occur more commonly on the right side. The increased occurrence on the right side is likely explained by the more cranial location of the right ovary, incisions not extending cranially enough, and therefore decreased visibility of the ovary and ligature placement. However, it has been suggested that the occurrence of ovarian remnant syndrome would be decreased with OVE because of the more cranial location of the incision, allowing greater exposure. One could also argue that during the OVE, due to making two cuts close to the ovary (pedicle and proper ligament) there is a higher chance of inadvertently leaving ovarian tissue. Unfortunately there currently are no studies evaluating the incidence of ovarian remnant syndrome in dogs undergoing OVH versus OVE. Ovarian remnant syndrome can be avoided with either surgery by proper visualization and correct surgical technique.

Stump granuloma. Ligatures of non-absorbable suture, poor aseptic technique, and/or large amounts of devitalized tissue can all cause inflammation and granuloma formation. One study (Okkens, 1981) found the incidence of ovarian pedicle granuloma in 6% of patients and at the uterine stump in 28%. Another report by the same author indicated that in patients with gynecologic complications after OVH, granulomas accounted for 15% of complications. Fistulous tracts associated with the granulomas occurred in 38% of dogs with granuloma formation. Suture associated granulomas can be prevented by using synthetic absorbable materials. Both OVE and OVH can result in the formation of granulomas from the ovarian stump, but only the OVH has the additional risk of for uterine stump granuloma formation.

Long-term complications

Endometritis and pyometra. Pyometra is defined as a hormonally mediated disorder in diestrus, resulting from interaction of bacteria and an endometrium that has undergone pathologic changes because of an exaggerated response to progesterone stimulation. Both cystic endometrial hyperplasia and pyometra are only seen in the presence of progesterone. This most commonly occurs during the luteal phase of the estrus cycle but can be induced by administration of exogenous progesterone. In one study from the University of Utrecht (Okkens, 1997), comparing the long-term effects of OVE versus OVH, none of the dogs had signs associated with endometritis or pyometra. In another study, also from the University of Utrecht, it was found that in dogs with gynecologic complications, 35% had stump pyometras, all of which had residual ovarian tissue. In that same study, there were 47 bitches that had histologic evidence of cystic endometrial hyperplasia, and all had residual ovarian tissue as well. When either OVH or OVE is correctly performed and all ovarian tissue is removed, and without exogenous progesterone, cystic endometrial hyperplasia or pyometra cannot occur.

Uterine tumor formation. The incidence of uterine tumors in the dog is reported as 0.4% of all canine tumors. Another study from the University of Pennsylvania found the incidence to be 0.03%. Among uterine tumors, 85-90% are benign leiomyomas and 10% are leiomyosarcomas. The risk for development of a malignant tumor in the uterus is calculated at 0.003%. The prognosis with leiomyomas

is excellent with complete resection. For leiomyosarcomas the prognosis is good with complete resection as long as there is no evidence of metastatic disease. To date there are no reports of uterine neoplasia in a dog that has had its ovaries removed prior to two years of age. This may suggest a hormonal influence in the development of these tumors. When deciding whether to perform OVE versus OVH, the surgeon must balance the risk for possible development of a tumor versus the development of surgical related complications from the procedure.

Mammary tumor formation. Mammary gland tumors are the most common tumors in female dogs, with a 3.4% incidence. Of these, 41-53% are reported to be malignant. The relative risk for malignant mammary gland tumor occurrence in dogs spayed before the first estrus is 0.5%. The relative risk increases to 8% between the first and second estrus, and 26% between the second and third estrus. Sterilization by either OVE or OVH before the first estrus will largely eliminate the risk of mammary tumor development in dogs.

Urinary sphincter mechanism incompetence. The occurrence of urethral sphincter mechanism incompetence (USMI) post-gonadectomy is the most common cause of urinary incontinence in spayed dogs. This has been reported to occur in 3-20% of spayed bitches an in only 0.2-0.3% of intact bitches. There is a large hormonal component to development of USMI, however the exact cause of USMI has not been completely identified and is likely multifactorial. Long-term studies have not detected any difference in the occurrence of incontinence between OVE and OVH dogs. Individual studies report an incidence of USMI ranging from 9-21% with OVE and 14-20% with OVH

Conclusion

Based on studies reported in the veterinary literature, and the unfortunately few prospective randomized studies, there is no strong scientific evidence for removal of the uterus during sterilization of the female dog. Ovariectomy provides an equally effective technique for sterilization in the dog and cat with no reported disadvantages. Since 1981, OVE has been the standard technique for sterilization of bitches at the University of Utrecht. No increases in short-term or long-term complications have been observed. The development of cystic endometrial hyperplasia and pyometra cannot occur if the ovaries are completely removed, and any occurrence of either is indicative of an ovarian remnant. The chances of malignant uterine tumor development is very low, at 0.003%, and therefore may not warrant the additional trauma of removal of the uterus. Potential advantages of the OVE include smaller incisions, better visualization of the ovarian pedicles, and the decreased risk of complications associated with decreased manipulation of the uterus. These advantages of OVE are magnified with the more novice surgeon.

Selected references

Berzon JL: Complications of elective ovariohysterectomies in the dog and the cat at a teaching institution: clinical review of 853 cases. Vet Surg 1979;8:89-91.

Burrow R, Batchelor D, Cripps P: Complications observed during and after ovariohysterectomy of 142 bitches at a veterinary teaching hospital. Vet Rec 2005;157:829-833.

McCarthy RJ, DeTora M: Ovariohysterectomy versus ovariectomy for elective sterilization of female dogs and cats: is removal of the uterus necessary? J Am Vet Med Assoc 2011;239:1409-1412.

Okkens AC, Kooistra HS, Nickel RF: Comparison of long-term effects of ovariectomy versus ovariohysterectomy in bitches. J Reprod Fertil Suppl 1997;(51):227–231.

Okkens AC, Dieleman SJ, van de Gaag I. Gynaecological complications following ovariohysterectomy in dogs, due to: (1) partial removal of the ovaries. (2) inflammation of the uterocervical stump [in Dutch]. Tijdschr Diergeneeskd 1981;106:1142-1158.

Peeters, ME, Kirpensteijn J: Comparison of surgical variables and short-term postoperative complications in healthy dogs undergoing ovariohysterectomy or ovariectomy. J Am Vet Med Assoc 2011;238:189-194.

Van Goethem B, Schaefers-Okkens A, Kirpensteijn J: Making a rational choice between ovariectomy and ovariohysterectomy in the dog: a discussion of the benefits of either technique. Vet Surg 2006;35:136-143.

Laparoscopy for reproductive surgeries: it's kinda' like playing Wii, but different

Roberto E. Novo
Columbia River Veterinary Specialists, Vancouver, WA

Objectives

- To discuss indications for laparoscopic reproductive surgeries in dogs and cats.
- To present different approaches and techniques for sterilization of female dogs.
- To describe the technique for laparoscopic exploratory for cryptorchid male dogs.
- To discuss the approach for canine vasectomy.

Key points

- Laparoscopy is a minimally invasive technique that can be used to sterilize female dogs and remove intra-abdominal testicles in cryptorchid males.
- Laparoscopy provides improved visualization, decreased postoperative pain and morbidity, and a faster return to normal activity.
- The use of laparoscopic vessel-sealing devices allows for improved surgical hemostasis and decreased surgical time.
- When preparing for a laparoscopic procedure, the surgeon always needs to be prepared to convert to an open approach if needed.

Overview

The use of laparoscopy in small animal surgery has increased in popularity in the past several years. However, it has not received widespread attention from veterinarians due to the cost of equipment, the requirement for training to master laparoscopic techniques, and the duration of laparoscopic procedures as compared to open procedures. With practice, the techniques can be easily mastered and surgical time quickly diminished. Relatively simple applications of laparoscopy are sterilization of female dogs and cats, and abdominal cryptorchidectomy in dogs and cats. Laparoscopy allows for excellent examination of the peritoneal cavity and either full laparoscopic surgery or laparoscopy-assisted procedures. Procedures are easier in larger patients, and may be combined with prophylactic gastropexy procedures in these patients which may also be at risk of gastric dilatation-volvulus.

The full laparoscopic techniques are not described in this presentation, only salient features. For full surgical detail, please see articles listed in the selected references section.

Instrumentation

For either laparoscopic sterilization or cryptorchidectomy, the equipment is the same and includes the laparoscope, light source, video imaging system, gas insufflators, and laparoscopic instrumentation (trochars, probes, forceps, etc.). A surgical table that is adjustable and tilts facilitates movement of the patient to improve visualization of the abdomen.

There are many hemostatic devices that have been used and proven effective for laparoscopic surgery. The use of extracorporeal sutures, hemoclips, bipolar/monopolar cautery, laser, and ultrasonic vessel-sealing devices are all available. In 2007, a prospective, randomized clinical trial was published, comparing the use of extracorporeal sutures, laparoscopic clips, and a bipolar vessel-sealing device for ligation of the ovarian pedicle during laparoscopy-assisted ovariohysterectomy (OVH). The bipolar vessel-sealing device was found to be associated with significantly shorter surgery times and a lower incidence of hemorrhage from the ovarian pedicle. It is therefore recommended that practices planning to offer laparoscopic procedures on a regular basis invest in a vessel-sealing device. These instruments are can seal vessels up to 7 mm in diameter, so are useful in all breeds, even large breeds. As an aside, vessel-sealing devices are also very useful in splenectomy procedures.

Laparoscopic ovariohysterectomy and ovariectomy

There has been a long lasting argument regarding whether or not the uterus should be removed during gonadectomy in female patients. In the USA, the preferred method is performance of ovariohysterectomy (OVH) whereas in most European countries, the ovariectomy (OVE) is the preferred method. In the USA, there has been a recent greater push to switch to the OVE procedure; however, much resistance is still present. Based on the evidence that is currently available in the veterinary literature, there is no reported benefit and therefore no indication for removing the uterus during routine neutering of healthy bitches. In a survey of the teaching programs among the veterinary schools in North America, the open OVH procedure is most commonly taught. However, if laparoscopy is performed for gonadectomy, most surgeons will perform OVE. When laparoscopy is performed, OVE is technically easier and quicker to perform. If there is pathology present in the uterus, then hysterectomy also should be performed.

There are a few contraindications for the use of laparoscopic gonadectomy in females. Absolute contraindications include the presence of a diaphragmatic hernia, septic abdomen, obesity, and performance in small patients (<2 kg). Although there are reports of successful pyometra surgery in the literature, rupture of the uterus is a significant risk. An open technique should be considered in these situations, or the surgeon should at least be prepared to switch to an open procedure if a laparoscopic procedure is initiated.

For each laparoscopy-assisted OVH, OVE, or cryptorchidectomy, multiple techniques have been described, using a number of ports (one, two, or three). The more common techniques use either two or three ports to allow placement of the laparoscope and instruments. Three ports are usually used for laparoscopy-assisted and completely laparoscopic OVH, whereas two ports are generally used for laparoscopic OVE. Regardless of the number of ports used, the surgeon should always have the patient clipped and prepared in the event that the procedure needs to be converted to an open laparotomy.

Laparoscopy-assisted ovariohysterectomy

A combined laparoscopic and open procedure is used with this technique. Laparoscopy is used to identify and transect the ovarian pedicle. The ovary is identified, grasped and pulled up to the body wall. A suture is passed through the body wall, directed through the proper ligament (not the ovary), and then passed back out of the body. The free ends of the suture are grasped with hemostats to secure the ovary in place. The bipolar vessel-sealing device or laparoscopic hemoclip is used to seal/ligate vasculature and sequentially break down the suspensory ligament, ovarian pedicle, and broad ligament on both sides. Sutures holding the ovaries are released. The ovaries and uterus are then exteriorized through the caudal port, with the incision being extended as needed. Once the ovaries and uterus are exteriorized, the ligation of the uterine arteries and uterine body are performed routinely. Excessive tension on the uterus during this portion of the procedure can result in tearing of the uterus, or hemorrhage. Care must also be taken while exteriorizing the ovary to avoid seeding of ovarian tissue into the peritoneal cavity. Once the caudal incision is closed, the pneumoperitoneum can be re-established and the camera can be re-inserted to evaluate for any hemorrhage from the uterine stump.

Laparoscopic ovariohysterectomy

The suspensory ligaments, ovarian pedicles, and broad ligaments are sealed/ligated as described above, but the uterine arteries and uterus are ligated within the abdomen. The uterine arteries can be individually ligated using a bipolar vessel-sealing device or hemoclips, and the uterine body can be ligated with a pre-tied loop suture or extracorporeal sutures. The bipolar vessel-sealing device can be used on smaller uteri. However, there is little advantage to ligating the uterus within the abdomen, especially in larger dogs, as it is more time consuming, and the caudal incision still has to be enlarged to allow removal of the ovaries and uterus. The laparoscopy-assisted OVH allows more secure ligature placement and is a speedier procedure, especially in large dogs or dogs with enlarged uteri.

Laparoscopic ovariectomy

Once the ovary is identified, the proper ligament is grasped and elevated. The bipolar vessel-sealing device or hemoclip is used to seal/ligate the vascular structures. The proper ligament, mesovarium, and ovarian artery and vein are transected sequentially. Small ovaries can be recovered through the lumen of one of the larger ports. In larger dogs or dogs with ovaries associated with significant adipose tissue, the instrument port may need to be enlarged to allow careful withdrawal of the ovary.

Ovarian remnant syndrome

Laparoscopy is also effective in identifying ovarian remnants from a previous spay procedure. Often the ovarian remnant is small, and located at the end of the ovarian pedicle. Adhesions may have developed at the ovarian pedicle, obscuring the ovarian remnant. The procedure is no different than laparoscopic ovariectomy, but usually only the ovarian pedicle needs to be ligated/sealed further down, closer to the abdominal wall. Tilting the patient to allow the abdominal organs to shift to the opposite side improves visibility of the ovarian pedicle region. Blunt probes may be used to move the duodenum or colon to allow the right and left pedicle, respectively, to be accessed.

Laparoscopic cryptorchidectomy

Cryptorchidism is thought to be a sex-linked autosomal recessive trait in dogs, so neutering and removal of the cryptorchid testicle is recommended. Torsion of the spermatic cord and testicular neoplasia are common pathologies that can develop in cryptorchid animals. One study described a 13.6 fold increase risk of testicular neoplasia in cryptorchid testes. The risk of torsion of the spermatic cord also is increased, with torsion often associated with testicular neoplasia.

The use of laparoscopy for cryptorchid castration allows for easy identification of the testicle within the abdomen with minimal trauma, postoperative patient discomfort, and wound related complications. In cases where localization of the cryptorchid testis is difficult, laparoscopic examination of the caudal abdomen and the openings to the inguinal rings provides excellent visibility. This view can help rule out the diagnosis of abdominal versus inguinal cryptorchidism. This approach may help minimize iatrogenic trauma to tissues attributed in some cases to poor visibility due to small paramedian approaches. Iatrogenic trauma to the ureters and/or urethra and inadvertent prostatectomy have been reported.

Preoperatively the side of the cryptorchid testis should be determined. Under heavy sedation or anesthesia the inguinal regions should be palpated. If a testicle is present within the scrotum, it can be gently manipulated cranially to determine from which side it originates. Cryptorchid testicles are usually abdominal or inguinal, or in rare instances are located within the inguinal canal. Palpation can be challenging in some cases, as often the cryptorchid testis is smaller than normal, and if present within the inguinal canal, then inguinal adipose tissue may make palpation difficult. Ultrasonography of the inguinal region or abdomen can be helpful in some cases to identify the location of the testicle. The patient should always be prepped for full abdominal surgery, in the event that the laparoscopic procedure needs to be converted to an open laparotomy. A two port laparoscopic technique is usually used for a laparoscopy-assisted cryptorchidectomy, whereas a three port technique is used for a complete laparoscopic cryptorchidectomy procedure. Instrument ports usually are introduced through the rectus abdominal musculature lateral to the prepuce.

With either approach, the caudal peritoneal cavity is first explored. Abdominal testes are often seen immediately upon entering the peritoneal cavity with the laparoscope. In cases where the testicle is not immediately visible, the areas of the inguinal rings are evaluated. If the spermatic cord and vascular pedicle are seen entering the ring, then the testicle is outside of the abdomen, likely in the inguinal region. If the gubernaculum is seen entering the inguinal ring, then the testis is within the abdomen, likely hidden by the bladder or other structures. Gentle traction of the gubernaculum will bring the testis into view.

Laparoscopic-assisted cryptorchidectomy

This technique offers a quick way to identify and exteriorize the abdominal testicle, allowing for easy ligation of the vascular pedicle and spermatic cord outside of the abdomen. Once the testicle is identified and grasped, it is extruded through the port. The port may need to be enlarged (separating along the muscle fibers of the rectus abdominal muscle) to allow exteriorization of the testicle. Once the testicle is outside the abdomen, a routine neuter can be done with ligation of the vascular pedicle and spermatic cord. If both testicles are within the abdomen, the opposite testicle can often be extruded from the same port as the first testicle. Alternatively a second port can be created on the opposite side. Once the caudal incision is closed, the pneumoperitoneum can be re-established and the camera can be re-inserted to evaluate for any hemorrhage.

Laparoscopic cryptorchidectomy

When performing the totally laparoscopic cryptorchidectomy, the vascular bundle and spermatic cord are ligated and transected within the peritoneal cavity. Once the testicle is identified, it is elevated towards the body wall. A bipolar vessel-sealing device is inserted through a second instrument port and used to seal and transect the testicular vascular pedicle, spermatic cord, and gubernaculums. In large dogs, the large pampiniform plexus should be either double sealed prior to transaction, or it should be sealed along its narrowest section below the plexus. Alternatively, hemoclips can be used for ligation. Extracorporeal ligatures can also be used, but this is more time consuming. Once the testicle is transected from its attachments, it is removed through one of the ports. The parapreputial port may need to be enlarged by separating along the muscle fibers of the rectus abdominal muscle. However if the subumbilical port along the linea is enlarged for removal of the testicle(s), there is less muscle trauma and pain that occurs as compared to the removal through the parapreputial port.

Laparoscopic cryptorchidectomy for torsion of the spermatic cord or neoplastic testes

Laparoscopy for this condition is more challenging, as often these testes are larger than normal size (as compared to an unaffected cryptorchid testis which is often smaller than normal). If the testis is larger than 8-10 cm, then an open approach should be considered. With laparoscopy a large incision needs to be done to allow exteriorization of the affected testis, so the benefit of a minimal approach is lost. As well, in many of these situations there are adhesions to adjacent structures (bladder, prostate, ureters, intestines, etc.), making laparoscopic removal technically difficult and prolonging surgery. In many situations, the neoplastic cryptorchid testis is small and mobile. Routine laparoscopic cryptorchidectomy can be done as described above, however the neoplastic testicle should be placed in a specimen retrieval bag before it is exteriorized to prevent seeding the port site with neoplastic cells.

Laparoscopic vasectomy

Although there may be an ethical debate as to whether or not vasectomies should be performed in dogs, there may be the rare situation where it makes sense (guard dogs or flock protection dogs, to prevent pregnancies but maintain behaviors). Obviously before performing a vasectomy in a dog, the owner should be counseled about the risks of testicular neoplasia, prostatic disease, perianal tumors, and perineal hernias. If a vasectomy is still indicated, a laparoscopic approach provides excellent visualization of the spermatic cord entering the inguinal ring. The cord is grasped and pulled away from the vascular bundle. It is recommended that a section of spermatic cord be removed. The bipolar vessel sealing device or hemoclips can be used to ligate and then transect a section (1-2cm) of the spermatic cord. This section can be removed via one of the instrument ports.

Postoperative care

With any of the procedures described above, patients need to be monitored for hemorrhage. Pain management is also imperative in these patients, however the duration of administration may be shorter than with open procedures. Owners should monitor incision sites for the usual redness, swelling, or discharge. Most complications following these procedures are associated with the incisions, including

seroma formation and dehiscence. If dehiscence occurs along the body wall, omental or intestinal herniation can occur. Because these patients may have less pain following their procedures and want to be active quickly, the owners should be well educated as to the importance of restricted activity, despite how well the patient feels. This of course is not new information for veterinarians, as we often have the same problem with open OHE procedures in young animals.

Selected references

Culp WTN, Mayhew PD, Brown DC: The effect of laparoscopic versus open ovariectomy on postsurgical activity in small dogs. Vet Surg 2009;38:811-817.

Davidson EB, Moll HD, Payton ME: Comparison of laparoscopic ovariohysterectomy and ovariohysterectomy in dogs. Vet Surg 2004;33:62-69.

Devitt CM, Cox RE, Hailey JJ: Duration, complications, stress, and pain of open ovariohysterectomy versus a simple method of laparoscopic-assisted ovariohysterectomy in dogs. J Am Vet Med Assoc 2005;227:921-927.

Gower S, Mayhew P: Canine laparoscopic and laparoscopic assisted ovariohysterectomy and ovariectomy. Compend Contin Educ Pract Vet 2008;30:430-440.

Hancock RB, Lanz OI, Waldron DR, et al: Comparison of postoperative pain after ovariohysterectomy by harmonic scalpel assisted laparoscopy compared with median celiotomy and ligation in dogs. Vet Surg 2005;34:273-282.

Mayhew PD, Brown DC: Comparison of three techniques for ovarian pedicle hemostasis during laparoscopic-assisted ovariohysterectomy. Vet Surg 2007;36:541-547.

Mayhew P. Laparoscopic and laparoscopic-assisted cryptorchidectomy in dogs and cats. Compend Contin Educ Pract Vet 2009;31: 274-281.

Miller NA, Van Lue SJ, Rawlings CA: Use of laparoscopic-assisted cryptorchidectomy in dogs and cats. J Am Vet Med Assoc 2004;224:875-878.

Schultz KS, Waldron DR, Smith MM: Inadvertent prostatectomy as a complication of cryptorchidectomy in four dogs. J Am Anim Hosp Assoc 1996;32:211-214.

That's not normal: how to handle vaginal anatomic anomalies

Roberto E. Novo
Columbia River Veterinary Specialists, Vancouver, WA

Objectives

- To discuss a variety of congenital and acquired vaginal diseases that can be seen in dogs and cats.
- To provide surgical treatment recommendations for various vaginal anatomic abnormalities.

Key points

- Fortunately many vaginal anatomic anomalies occur rarely, however prompt identification and recommendations are necessary to minimize morbidity to the patient.
- Abnormalities in embryonic canalization of the paramesonephric duct between the end of the Müllerian duct and the urogenital sinus can cause many variations of anatomic abnormalities, including hypoplasia, atresia and septa of the vagina, or abnormal connections between the vagina/vestibule and rectum/anus.
- To date there is no definitive documentation that sterilization prior to the first estrus will cause vulvar hypoplasia (juvenile/recessed vulva) in dogs. However sterilization prior to the first estrus will decrease the risk of mammary tumor development in dogs.
- Clitoral hypertrophy most commonly occurs in dogs with intersex disorders, hyperadrenocorticism, or after having received anabolic steroids. Dogs with intersex disorders will often have ovotestes in place of one or both ovaries.
- Vaginal septa or bands may be digitally broken down or surgically removed. Vestibulovaginal stenosis may be identified in many patients, but clinical significance and the need for surgical correction depends on severity and clinical signs.
- Vaginal edema must be differentiated from vaginal prolapse. Vaginal edema involves prolapse of edematous mucosa on the floor of the vaginal vault. Vaginal prolapse is a circumferential prolapse of the vagina and often includes the cervix.
- Of the reported vaginal neoplasias, 73-94% are benign, with leiomyomas being the most common, and appear to be sex hormone dependent.

Overview

There are a number of developmental and acquired conditions affecting the vagina and vulva that can be seen in both dogs and cats. Most commonly, dogs will be affected with these aberrations. Congenital conditions, which primarily affect younger dogs and cats, include rectovaginal fistula and anovulvar cleft; vulvar/vaginal hypoplasia and atresia; clitoral enlargement; and vaginal septa, bands, or stenosis. Acquired conditions generally affect older animals and include vaginal neoplasia and vaginal prolapse. However, some acquired conditions such as vaginal hyperplasia and perivulvar dermatitis can affect younger dogs. It is important to note that some acquired problems may develop secondary to abnormal anatomy of the reproductive tract so a thorough examination of the reproductive system is imperative in these animals.

Anovulvar cleft

Anovulvar cleft is a rare defect that occurs as a result of inappropriate fusion of the urogenital folds, with a cleft or trough present between the anus and vulva. This abnormality can be observed in sexually normal females or those with intersex disorders. The vestibular floor and clitoris are exposed, resulting in fecal contamination and hyperemia. If not corrected, this abnormality will cause increased occurrences of urinary tract infections due to fecal contamination, inflammation, and constant licking by the patient. This defect is easily corrected with a perineoplasty, and provides a good cosmetic appearance. Surgical correction is done by creating an H-shaped or inverted V-shaped incision along the mucocutaneous junction of the cleft. The skin and mucosal edges are separated, allowing the submucosa

and skin to be closed primarily across the midline of the perineum. The vestibular mucosa is then apposed to the skin edges to recreate the dorsal wall of the vestibule/vulva.

Rectovaginal fistula

Patients with a rectovaginal fistula, or connection between the rectum and vagina, will often also have some degree of atresia ani. In these patients fecal material is excreted through the vulva. Severity of clinical signs (tenesmus, distended abdomen, bulging perineum) depends on the degree of anal atresia (or stenosis), type of diet, and size of the fistula. In one study evaluating puppies and kittens with atresia ani, 12 animals were identified, and eight of them had rectovaginal fistulas. Dogs with atresia ani may have megacolon as a complicating factor. Vaginography or a barium sulfate enema can be used to demonstrate the location and size of the fistula. Correction of the rectovaginal fistula is usually simple, with the difficulty being addressing of the atresia ani. The anal opening is reconstructed by in situ anoplasty, full anal reconstruction, or balloon dilation to allow normal fecal excretion through the anus. The rectovaginal fistulous tract is then resected and both the vaginal and rectal walls are closed. In severe cases of atresia ani (type III - rectum ends in blind pouch with no anal structures), fecal incontinence may be a long-term problem.

Vulvarhypoplasia

Vulvar hypoplasia (also called recessed/juvenile/infantile vulva) frequently occurs in spayed females. To date there is no definitive documentation that sterilization prior to the first estrus is the cause for this abnormality. Theoretically it makes sense that development of the vulva may be affected without hormone stimulation, however occurrence of vulvar hypoplasia has been reported in intact bitches. Waiting for the first heat to allow for vulvar maturity has been proposed, but there is no evidence that letting a bitch go through a heat cycle is associated with long term increase in vulvar size. However the risk for development of mammary cancer increases significantly increases after the first heat.

Dogs with vulvar hypoplasia often present for perivulvar dermatitis, which is often exacerbated by persistent moisture from urine within the skin perivulvar skin folds. Perivulvar dermatitis is also seen in obese dogs, as the vulvar cleft becomes tucked underneath the more prominent perineal skin folds. Recurrent vaginitis and urinary tract infections are very common in these patients as well. Management of these patients generally involves weight loss and medical and surgical management. Gentle cleansing of the skin folds (benzoyl peroxide shampoo, topical astringent, antibiotic-steroid creams) will help minimize local inflammation prior to surgery. Once the initial inflammatory process is controlled, then an episioplasty is recommended.

An episioplasty (vulvoplasty) is performed to remove the excess perivulvar skin folds and the underlying subcutaneous fat. The goal is to remove enough skin so that the vulva is no longer recessed without creating excessive tension on the incision site. Excessive subcutaneous fat dorsal to the vulva is also removed, which is critical in obese animals. The amount of skin to be removed is assessed by elevating the skin folds and assessing expected tension. Two crescent-shaped skin incisions are made around the vulva. The outlined skin and subcutaneous tissues are removed, allowing for closure of the incision. Initial apposition at 3, 9, and 12 o'clock is recommended to determine if adequate amount of skin has been removed. Additional skin can be removed from the outer margin if the vulva is still recessed or the skin folds persist. If the vulvar folds are effectively removed, then dermatitis is usually resolved; however, persistent urinary incontinence may be present. One study (Hammel, 2002) showed that the incidence of urinary incontinence was reduced by vulvoplasybut it remained as the most common residual sign after surgery. In that study, the incidences of urinary tract infection (UTI), vaginitis, and external irritation were greatly improved following surgery.

Vaginal hypoplasia and atresia

Abnormal development of the vagina is rarely reported in dogs and cats. Abnormalities in embryonic canalization of the paramesonephric duct between the end of the Müllerian duct and the urogenital sinus can cause variations of these abnormalities. In sterilized females, vaginal hypoplasia

may never be identified; however, in a breeding bitch or queen, infertility, painful breeding, or dystocia may be the presenting problem. With vaginal atresia, the patient would present for suspected 'pyometra' due to a dilated/distended uterus from accumulation of normal secretions. Routine ovariohysterectomy should be recommended for any patient with these abnormalities. Although there is no information regarding the genetics of these abnormalities, breeding of animals with these traits is not encouraged.

Clitoral hypertrophy/os clitoridis

Hypertrophy of the clitoris most commonly occurs in dogs with intersex disorders, hyperadrenocorticism, or after having received anabolic steroids. This condition can also be observed in normal females. The clitoris often protrudes through the vulvar cleft and may contain an os clitoridis. Clinical signs most often involve excessive licking of the vulva due to clitoral irritation from exposure. These patients may be presented by owners for cosmetic reasons. Withdrawal of anabolic steroids and treatment for hyperadrenocorticism may resolve the clitoral hypertrophy. Gonadectomy is recommended in these patients. The presence of an abnormal uterus and ovotestes is common in these animals. If an os clitoridis is present, clitoral enlargement may not resolve with gonadectomy. In these cases amputation of the clitoris is recommended. Clitoral amputation is performed by simple submucosal dissection and mucosal apposition. Dogs with intersex disorders may have significant bleeding during dissection because of the presence of erectile tissue. An episiotomy may assist with visualization.

Vaginal septa, bands and stenosis

A number of vaginal and vestibular congenital abnormalities occur as a result of imperfect joining of the genital folds, genital swellings, or Müllerian ducts. These conditions may be incidental findings on a physical examination. Females with stenosis or bands may present with clinical signs of chronic vaginitis or recurrent UTI, which may be associated with urine pooling in the anterior portion of the vagina. Others may present for unsuccessful attempts or painful natural breeding. Digital and visual examination of the vagina is imperative in these patients. Evaluation for other abnormalities of the genitourinary system is recommended, as vaginal bands are frequently associated with ectopic ureters in dogs.

A persistent or imperforate hymen and small vaginal bands can be corrected with digital breakdown of the membrane. More fibrous vaginal bands and vaginal septa may require an episiotomy and surgical resection. Depending on the extent of the mucosal defect remaining after surgical removal of the band or septa, the defect can be left to heal by second intention. If a significant band is resected, mucosal apposition is important to prevent significant scar tissue formation or fibrous healing to the opposite mucosal wound.

If vaginal palpation identifies the presence of stenosis, a contrast vaginogram can be performed to further evaluate the severity of stenosis. A stenosis to vaginal ratio can be classified as follows: normal - > 0.35; mild - 0.26-0.35; moderate - 0.25-0.20; and severe - < 0.20. This ratio is calculated by dividing the height of the vestibulovaginal junction by the maximum height of the vagina on a lateral vaginourethrogram. Cases with vestibulovaginal stenosis can have two types of surgical procedures, depending on the severity of the stenosis. Cases with moderate to severe stenosis may benefit from a vaginal resection and anastamosis, whereas those with either mild or moderate stenosis may only need a T-vaginoplasty.

The recommmendation to perform surgery on these cases is debatable, however addressing those patients with severe stenosis may be advantageous. One study (Crawford, 2002) evaluated the influence of vaginal stenosis, pelvic bladder, and recessed vulva on the response to treatment for clinical signs of lower urinary tract disease in dogs and found that vestibulovaginal stenosis is an important factor in dogs with ratios < 0.20. It is recommended that vaginectomy or resection and anastomosis should be considered in these cases (Kieves, 2011). Subjectively, if the vaginal mucosa (cranial to the stenosis) is more severey inflamed than the vestibulular mucosa (caudal to the stenosis), then it is likely that the vaginal stenosis may be playing a role in the chronic inflammation. A vaginal resection and anastamosis is technically difficult due to the close proximity of the urethral papilla to the stenosis. The stenosis is

approached via a perineal incision between the anus and vulva, and does not require an episiotomy. This surgery can be easily combined with a vulvoplasty procedure. A complete vaginectomy is the final option for correction of vaginal stenosis in bitches that are not intended for breeding.

Vaginal prolapse

Vaginal prolapse includes two very different disease processes, although both with similar underlying etiology. Both conditions are estrogen dependent and develop during prosetrus or estrus. One process involves prolapse of edematous mucosa on the floor of the vaginal vault, referred to as *vaginal edema*. The second process involves a true prolapse of the vagina, in which the prolapse is circumferential and often includes the cervix. These dogs have *true vaginal prolapse*. For ease of discussion, the terms *vaginal edema* and *true vaginal prolapse* are used when discussing the two disease processes.

Vaginal edema

Vaginal edema (previously referred to as vaginal hyperplasia) involves prolapse of edematous mucosa on the floor of the vaginal vault. Vaginal edema occurs in young intact bitches in proestrus or estrus; this condition has not been reported in queens. Brachycephalic and large breeds are overrepresented. This disease has a familial predisposition, so breeding of these patients is not recommended. This condition must be differentiated from a neoplastic process.

Normally, during the follicular phase of the estrous cycle, the vaginal and vestibular mucosa becomes thickened and edematous. Occasionally an exaggerated response occurs, resulting in excessive edema. The submucosal tissue edema and redundant mucosa at the floor of the vagina just cranial to the urethral tubercle can create a perineal bulge or protrude through the vulvar labia as a fleshy red mass. In these cases the edematous tissue has been present for some time within the vestibule, and may go unnoticed as a simple bulging of the perineum. Continued growth, movement, and inflammation can cause sudden exteriorization between the vulvar folds. Although the mass can appear large, the base is often small (1-2 cm wide stalk). Often owners call in a panic reporting that this mass suddenly appeared. On examination the tissue may be traumatized due to exposure, desiccation, and self-mutilation. This condition can be differentiated from vaginal prolapse as the urethral tubercle is in its normal position. The vaginal edema usually develops just cranial to the urethral tubercle, so it is visible on evaluation of the base of the mass.

Conservative management consists of protection of the exteriorized tissue with lubricants and prevention of self-mutilation. Once the patient is out of heat, the vaginal edema regresses spontaneously during the luteal phase. However, recurrence is common during subsequent estrous cycles. Recurrence can also occur at parturition, resulting in dystocia. Gonadectomy is curative and should be considered to prevent recurrence. Surgical resection of the mass should be considered if the bitch is intended for breeding or if the tissues are significantly exposed or traumatized. Surgical removal of the mass alone will not prevent recurrence in subsequent cycles. A standard episiotomy is performed to expose the base of the edematous tissue. The mass is lifted off of the vestibular floor to visualize the urethral opening. The urethra is catheterized to prevent iatrogenic trauma during surgery. A transverse elliptical incision is made around the base, removing the abnormal vaginal tissue, and the vaginal mucosal defect is apposed carefully avoiding the urethral orifice. Significant bleeding is often associated with surgical removal.

True vaginal prolapse

True vaginal prolapse occurs less frequently than vaginal edema and also is associated with normal estrus. Prolapse can be either partial or complete, with the cervix being exteriorized with a complete vaginal prolapse. In both cases there is a doughnut-shaped eversion of the edematous vaginal tissues. This is differentiated from vaginal edema in that there is circumferential involvement of the vaginal mucosa and the urethral tubercle, with a central lumen.

As with vaginal edema, no treatment may be necessary, as patients with mild prolapse can have spontaneous regression during diestrus. More severe prolapses may require protection of exposed tissues

and/or replacement of the prolapsed mucosa. Under general anesthesia, the everted tissue is cleaned with a dilute antiseptic solution or saline. With severe tissue edema, application of 50% dextrose solution to the mucosal surface may decrease its size, facilitating reduction. Digital manipulation or a lubricated plastic syringe can be used to reduce the tissues. An episiotomy may be necessary to provide better exposure for reduction. Reduction can also be assisted by traction on the uterus via a ventral abdominal approach, especially if the patient is intact and an ovariohysterectomy is being performed at the same time. Once the vagina is reduced, re-prolapse can be minimized by suturing the uterine body or the broad ligament to the abdominal wall. If an abdominal approach is not performed, reduction can be maintained by placing mattress sutures between the vulvar lips. A urinary catheter may need to be maintained until the vaginal swelling subsides.

More chronic prolapses with secondary necrosis, infection, or hemorrhage of the prolapsed tissues may require surgical resection of the devitalized tissues to prevent further sepsis and self-mutilation. As well, these patients need to be fully evaluated to treat for any underlying sepsis, hypotension or anemia. Due to the severity of inflammation of these chronic prolapses, an episiotomy helps with exposure and placement of a urinary catheter. A stepwise full-thickness circumferential incision is made in the vaginal wall, with horizontal mattress sutures used to close the incision edges. This is continued circumferentially in small sections until the entire prolapsed tissue is resected.

Vaginal neoplasia

Vaginal tumors are uncommon in dogs and cats, reported in 0.85-3% of females with tumors. Of the reported vaginal neoplasms, 73-94% are benign, with leiomyomas being the most common. Leiomyomas appear to be sex hormone dependent and occur most frequently in nulliparous intact dogs (Kydd, 1986). Leiomyomas originate from the vaginal wall and are often intraluminal and pedunculated. Based on these findings, most benign tumors are amenable to local resection via episiotomy. Intact bitches should be spayed at the same time to prevent recurrence. Malignant tumors are less common, with leiomyosarcomas and transmissible venereal tumors being the common types. Malignant tumors tend to be more infiltrative and have a wide base. Leiomyosarcomas are more likely to extend into the extramural regions. Malignant tumors often require more extensive surgical resection, with total or subtotal vaginectomy being required.

Depending on the location and extent of the vaginal mass, resection may be performed via a perineal approach, abdominal approach, or combined approach. A pubic osteotomy may be required in cases where significant adhesions and perivaginal tissue involvement is present. A recent report (Nelissen 2012) described a combined caudal abdominal and vestibular approach for subtotal vaginectomy in 11 dogs (six were combined with an ovariohysterectomy). This approach allowed complete resection of extensive vaginal lesions, with no major complications, and favorable outcomes. Those patients with benign disease had better prognosis than those with malignancies.

The abdominal approach was used to carefully dissect the fascial and peritoneal attachments between the vaginal tissue and the rectum and urethra. The cranial and caudal branches of the vaginal artery and vein were ligated and the perivaginal tissues were dissected as far caudally as possible. A transfixation suture with a large loop was anchored through all layers of the cranial vaginal opening, and passed into the vaginal lumen. Once the ventral celiotomy was closed, the patient was placed in sternal recumbency. Via a midline episiotomy approach, the loop within the vaginal lumen was identified and retracted caudally, inverting the cranial aspect of the vagina into the episiotomy. Resection of the vagina just anterior to the urethral papilla was then performed. The vestibule was closed just anterior to the urethra and the episiotomy was closed routinely. A urinary catheter should be maintained throughout the procedure to help identify the urethra during dissection, and postoperatively to allow swelling to subside.

Regardless of the method of resection, in cases of malignant neoplasia, follow-up treatments may be required to minimize or slow down recurrence due to invasion into perivaginal tissues. With benign tumors, even if aggressive resection is required, prognosis is good.

Selected references

Crawford JT, Adams WM: Influence of vestibulovaginal stenosis, pelvic bladder, and recessed vulva on response to treatment for clinical signs of lower urinary tract disease in dogs: 38 cases (1990-1999). J Am Vet Med Assoc 2002;221:995-999.

Hammel SP, Bjorling DE: Results of vulvoplasty for treatment of recessed vulva in dogs. J Am Anim Hosp Assoc 2002;38: 79-83.

Hedlund CS: Surgery of the reproductive and genital systems. In: Fossum TW, editor. Small animal surgery. St. Louis: Mosby; 2002. p. 610-648.

Kieves NR, Novo RE, Martin RB: Vaginal resection and anastomosis for treatment of vestibulovaginal stenosis in 4 dogs with recurrent urinary tract infections. J Am Vet Med Assoc 2011;239:972-980.

Kydd DM, Burnie AG: Vaginal neoplasia in the bitch: a review of forty clinical cases. J Small Anim Pract 1986;27:255-263.

Novo RE: Surgical repair of vaginal anomalies in the bitch. In: Bonagura JD, Twedt, DC, editors. Kirk's current veterinary therapy XIV. St. Louis: Saunders Elsevier; 2009. p. 1012-1017.

Mathews KG: Surgery of the canine vagina and vulva. Vet Clin North Am Small Anim Pract 2001;31:271-290.

Pettit GD: Vagina and vulva: surgical treatment of vaginal and vulvar masses . In: Bojrab MJ, editor. Current techniques in small animal surgery. Baltimore: Williams & Wilkins; 1998. p 503.

Rahal SC, Vicente CS, Mortari AC, et al : Rectovaginal fistula with anal atresia in 5 dogs. Can Vet J 2007;48:827-830.

If I tell you ... I'll have to kill you: tricks, tips, and ideas for common reproductive procedures

Roberto E. Novo
Columbia River Veterinary Specialists, Vancouver, WA

Objectives

- To present techniques to help differentiate and solve common problems during common reproductive surgical procedures.

Key points

- If you are struggling to see the ovary, uterus, and/or cryptorchid testicle, then extend the surgical incision. You want to make sure to clip and prep for a large abdominal approach.
- Complete agenesis of the ovaries and uterus or testicles are rare occurrences, with more commonly an abnormal, atrophied vestigial structures (mesonephric/Müllerian ducts) identified in their place.
- In patients with uterine hypoplasia, uterus unicornis or with segmental agenesis, both ovaries are usually present, so the second ovary must be searched for and removed.
- Using a miller's knot for ligation of the ovarian stump can be useful in overweight animals.
- Using the "biological retractors" improves your ability to find the ovary and uterus, as the mesoduodenum/mesocolon will 'retract' the intestines from your field of view.
- If a patient comes back into heat after the ovaries have been removed, the most likely cause is that all of the ovarian tissue was not removed.
- To identify an abdominal cryptorchid testicle, follow the ductus deferens from the bladder or the gubernaculum from the inguinal ring, and the testicle should be at the end.

Overview

Although sterilization of dogs and cats are common procedures done at veterinary practices every day, they can be associated with frustration and stress, as not every procedure goes as expected. When the procedure does not go routinely, it often results in increased surgical and anesthetic time for the patient, as well as an increased risk of postoperative complications. Difficult surgeries often occur in patients that are overweight, very young patients where the tissues are more friable, and those with congenital abnormalities where the normal structures are not present or appear abnormal. Higher complications also can occur in patients where the surgical approach is small, so complete identification and evaluation of the structures being removed cannot be appropriately made.

Surgical approach

In my experience, the key to minimizing many of the complications associated with sterilization surgery (ovariectomy [OVE], ovariohysterectomy [OVH], and cryptorchidectomy), is a good surgical approach to permit exposure. This allows the surgeon to fully evaluate structures correctly for removal, evaluate ligature placement, and evaluate for any hemorrhage. For ANY abdominal procedure, the surgeon should make sure that the patient is clipped and prepared for a large abdominal approach. The patient should be draped for a large approach, even if the surgeon chooses to make a smaller approach. In overweight animals there will be increased adipose tissue within the ovarian pedicle, making ligation more difficult, so the approach may need to be extended further cranially to allow all structures to be seen. When abnormal anatomy is present, then a larger surgical approach is necessary to fully evaluate the reproductive tract. Good knowledge of the anatomy is required in those cases where structures are absent or not in the normal location.

The umbilicus is often recommended as the cranial limit for the abdominal incision when performing an OVH. However, there is no anatomic relationship between the umbilicus and the location of the ovaries. The cranial and caudal limits of the abdominal incision for an OVH should provide an

incision of sufficient length to easily and safely remove the ovaries and uterus and to easily and safely ligate the ovarian and uterine vasculature.

Parapreputial approaches are often utilized for cryptorchidectomy procedures. This means that the skin incision is parapreputial , however then the penis is retracted to the side and the abdomen is approached via a midline linea alba incision. If the parapreputial approach is continued through the musculature (rectus abdominus muscle), there will be significant bleeding, tissue trauma, poor visualization, and postoperative pain.

Ovariectomy/ovariohysterectomy

When performing an OVE/OVH, the ovary should be present anywhere from below the kidney to the pelvic canal. The uterus, if present, is always located between the colon and bladder. When the surgeon is having difficulty identifying normal structures, then the first thing to do is to extend your incision. Visibility is critical to help identify structures without causing tissue damage.

Using the "biological retractors" improves your ability to find the ovary and uterus, as the mesoduodenum/mesocolon will 'retract' the intestines from your field of view. On the right side find the descending duodenum and reflect it to the left exposing the caudal pole of the right kidney, the right ovary and right uterine horn. On the left side the descending colon, reflect it to the right exposing the caudal pole of the left kidney, the left ovary and left uterine horn. Alternatively the bladder can be retroflexed and the uterine body identified between the bladder and colon.

Congenital abnormalities of the reproductive tract are not common, with minimal information in the veterinary literature. These abnormalities can present difficulties for veterinarians when found incidentally during OVH or OVE. In the female, the more common abnormalities include uterine hypoplasia (poorly developed uterus or uterine horn), uterus unicornis (complete agenesis of one uterine horn), and segmental agenesis (an underdeveloped or absent portion of a uterine horn). In 2010, McIntyre et al reported that congenital anomalies of the uterus were identified in 0.09% (49/53,258) of female cats and 0.05% (15/32,660) of female dogs. Uterine anomalies identified included unicornuate uterus (33 cats and 11 dogs), segmental agenesis of 1 uterine horn (15 cats and 3 dogs), and uterine horn hypoplasia (1 cat and 1 dog). Ipsilateral renal agenesis was present in 29.4% (10/34) of cats and 50.0% (6/12) of dogs with uterine anomalies in which kidneys were evaluated. Mummified ectopic fetuses were identified in 4 cats with uterine anomalies. Both ovaries and both uterine tubes were present in most animals with uterine anomalies.

So when performing a spay and discovering that one uterine horn is absent or abnormal you must search for the ovary that is associated with that uterine horn. If a broad ligament is present, then it can be followed cranially to the ovary. If no broad ligament is present on the involved side, use of the biological retractors will help localize the ovary, just caudal to the kidney.

In female cats, if no ovarian or uterine structures are identified after persistent exploration of the abdomen, the odds are that you are spaying a male cat! Have someone check under the drape.

Dropped pedicle/hemorrhage

Standard technique for ligation of the pedicle is a three clamp technique. The number of clamps used is not as critical as the security of the ligature. The ligature should be placed in the 'crush' zone of a clamp. The hemostat allows the adipose tissue in the crush area to be pushed out, leaving only vasculature and connective tissue within the ligature. When using multiple clamps, a common mistake is placing a ligature too close to a second clamp. The clamp will keep the tissues spread apart, and not allow the ligature to be tightened securely. Often this is noted when the second clamp is released and the ligature is loose, or when the pedicle is transected and bleeding occurs. To prevent this, the second clamp can be 'flashed' when placing the ligature close by. The clamp is released, the ligature is tightened, and then the clamp is reclosed.

Ligatures can be tied with a square knot, a surgeon's knot, or a miller's knot depending on the size of the pedicle and the preference of the surgeon. Of these, the miller's knot is the most secure especially in patients with large, fatty ovarian pedicles.

If an ovarian pedicle tears, retracting back into the abdominal cavity prior to ligation, you must retrieve and ligate the pedicle. Each pedicle must be checked for any bleeding prior to closure as well. Observation of the pedicle is the best way to assure no bleeding is present. To check the ovarian pedicle or retrieve a dripped pedicle, using the "biological retractors" improves ability to see the pedicles. On the right side, find the descending duodenum and reflect it to the left exposing the caudal pole of the right kidney, the right ovary and right uterine horn. On the left side the descending colon, reflect it to the right exposing the caudal pole of the left kidney, the left ovary and left uterine horn. To evaluate the uterine body for bleeding, the bladder can be retroflexed to visualize the tied ligated uterus between the bladder and colon.

Although bleeding from a dropped pedicle can be stressful, the patient will not exsanguinate before your eyes. Depending on the size of the surgical incision, it may need to be extended to better evaluate the affected pedicle. Once the pedicle is identified, then it can be either exteriorized with two fingers or with forceps grasping the tissue where the bleeding is occurring. There is absolutely no need to reach in with forceps and start grasping large sections of retroperitoneum randomly in the pedicle area, as this will increase the chances of damaging the ureter inadvertently. Care should be taken if gauze squares are used to clear the blood from the area. Know how many gauze squares you are putting in and taking out. Once the pedicle is exteriorized you can place two hemostats and ligate in the crushed area of the most proximal (deep) hemostat. Alternatively, if there is not much tissue present, you can place a miller's knot around the pedicle to control the bleeding, and then place your circumferential ligature in the crushed area from the hemostat.

Ovarian remnants

Ovarian remnant syndrome (ORS) is defined by the presence of functional ovarian tissue in a previously spayed bitch. An ovarian remnant occurs when ovarian tissue is left in the abdomen after an OVE or OVH. Recurrence of estrus following OVH has been reported in 17-43% of dogs. The most common cause for this occurrence is either crushing part of the ovary or cutting part of the ovary off with the ovarian pedicle during removal of the ovary. Ovarian tissue could also be dropped into the abdomen inadvertently (during laparoscopy). The ovarian tissue can revascularize and actively secrete hormones, causing patients to exhibit signs of estrus again. This can occur days, weeks or years after the original procedure. Confirmation of the patient being in estrus should be done via vaginal cytology. If this is consistent with estrus, then further bloodwork may not be necessary. However, a serum luteinizing hormone (LH) assay can be done. If LH concentration is high, no estrogen is present. If the LH is low, there is estrogen present, and without an exogenous source, then ovarian tissue must be present.

If a patient comes back into heat after the ovaries have been removed, the most likely cause for this is that ovarian tissue remains. There are no reports in the veterinary literature of true ectopic ovaries. Accessory ovarian tissue can be small and have been reported in cats to be located in the proper ligament of the ovary but separated by connective tissue from the normal ovary. If the normal ovary is removed, the accessory ovary may become functional. One study published in 2010 evaluated patients presenting for ORS. The most common clinical signs were those associated with proestrus and estrus. More dogs than cats were affected, and all residual ovarian tissues were found in the region of the ovarian pedicles. The right ovary in dogs was affected significantly more often than the left ovary. Seven animals had neoplasms of the reproductive system. These animals had a significantly longer interval between OVH and diagnosis of ORS than did the 14 animals without neoplasms. Surgical removal of residual ovarian tissue resulted in resolution of clinical signs. Based on these findings, ORS is usually a result of surgical error during an OVH or OVE procedure and surgical exploration and removal of the ovarian remnant is recommended.

To avoid this complication, make sure you have fully exteriorized the ovaries. In order to avoid clamping part of the ovary off, either fully observe the ovary or have a thumb and index finger on the ovary so you can feel where the ovary ends, allowing safe placement of your surgical clamps. Always examine the transected tissue, opening the bursa to make sure you have the entire ovary. In cats, the ovary is not in a bursa. This should be done on every OVE and OVH procedure.

If a functional ovarian remnant is present, you must surgically remove it. Performing the surgery while the animal is in heat will make locating the remnant easier. Use of the "biological retractors" for exposure and grasping with fingers are the best methods to expose and exteriorize the ovarian pedicles and find the ovarian remnant. Once the remnant is exteriorized, place two clamps proximal to the remnant, and ligate in the crushed area of the most proximal clamp.

When exploring the abdomen, both sides should be evaluated, although the right side is the most common location. Palpate the tissue in the area for a firm mass or a full ovary. If no obvious tissue is identified, both ovarian pedicles should be resected. Careful evaluation of the rest of the abdomen should be done as well in the event that a section of ovary was dropped during the initial procedure. Often the remnant is easily identified. However, provide a good surgical approach to allow adequate ability to see all abdominal contents and always allow plenty of time to thoroughly evaluate the abdomen. It is also recommended to send the tissue that was removed for histopathologic evaluation to confirm the resected tissue is ovarian in origin. Some surgeons like to explore during estrus in hopes that increased vasculature makes remnants more visible. Others like to administer human chorionic gonadotropin during estrus in order to cause ovulation, in hopes that luteal tissue will be present and the remnant more easily identified.

Cryptorchid testicle

Testicular agenesis is an extremely rare condition in dogs or cats, so if a testicle is not in the scrotum, the odds are that the patient is cryptorchid. One study described a 13.6 fold risk of testicular neoplasia in cryptorchid testes. The risk of torsion of the spermatic cord also is increased, with affected testes often being neoplastic. Due to these risks, removal of cryptorchid testicles is always indicated.

Preoperatively, the side of the cryptorchid testis should be determined. Under heavy sedation or anesthesia the inguinal regions should be palpated. If a testicle is present within the scrotum, it can be gently manipulated cranially to determine from which side it originates. Cryptorchid testicles are usually abdominal or inguinal. Very rarely, a testis is retained within the inguinal canal. Palpation can be challenging in some cases, as often the cryptorchid testis is smaller than normal, and if present within the inguinal canal, then inguinal adipose tissue may make palpation difficult. Ultrasonography of the inguinal region or abdomen can be helpful in some cases to identify the location of the testicle. In cats, if a retained testicle is suspected, the simplest way to determine if testosterone is present is to check for testosterone-dependent penile spines. Penile spines will atrophy by six weeks following complete castration.

For inguinal cryptorchid testicles, an incision is made over the testicle and a routine neuter is performed. For abdominal cryptorchid testicles, a standard midline/parapreputial approach entering the abdomen via a linea alba incision is recommended. The cryptorchid testicle is usually very mobile and can be easily exteriorized. Full parapreputial approaches through the rectus muscle for unilateral cryptorchidectomy are discouraged due to poor ability to see abdominal content, increased bleeding, and increased soft tissue trauma and pain. Inadvertent prostatectomy due to decreased ability to see and identify abdominal structures has been described in the literature. When performing a cryptorchidectomy, the abdominal testicle should be present anywhere from below the kidney to the inguinal ring. If the spermatic cord and vascular pedicle are seen entering the inguinal ring, then the testicle is outside of the abdomen. If the gubernaculum is seen entering the inguinal ring, then the testis is within the abdomen, and gentle traction of the gubernaculum will bring the testis into view. The bladder can also be retroflexed, and the ductus deferens located dorsal to the neck of the bladder. The ductus can be followed to the testicle.

If the testicle is truly not identified during surgery and ruling out monarchism or anarchism is desired, then a gonadotropin releasing hormone stimulation test will tell you if there is functional testicular tissue anywhere in the dog.

Hermaphrodite/pseudohermaphrodite

Disorders of sexual development are not straightforward. A "true" hermaphrodite has the presence of both gonads (ovarian tissue and testicular tissue). The gonadal tissue can be present as a complete ovary, complete testicle or any variation, such as an ovotestes. This definition is used regardless of the presence of either male, female or both external genitalia.

An animal is termed a "pseudo" hermaphrodite when the phenotype (external genitalia) does not match the gonadal tissue (ovary or testicle). These are further classified as either a "male" or "female" pseudohermaphrodite based on the gonadal tissue that is present. A male pseudohermaphrodite has the external genitalia of a female and testicular tissue present (often abdominal and not apparent externally). A female pseudohermaphrodite has the external genitalia of a male and ovarian tissue. These patients can also have ambiguous external genitalia but are still named by the gonadal tissue present.

Finding a testicle in the place of an ovary can certainly be confusing, however, bottom line, when sterilizing a patient, be it a male or a female organ, it needs to come out. If you are searching for a cryptorchid testicle and find a hypoplastic uterus and an ovary, then proceed to perform an OVH. Most patients will still have two gonads (male, female, or one of each), so removal of both should be done as you normally would for that particular gonad. If you are going to spay a patient with a prominent os clitoris, then be prepared to find testicular tissue within the abdomen. These situations are the reason that you always want to have your patient clipped and prepped for a more extensive approach.

Selected references

Ball RL, Birchard SJ, et al: Ovarian remnant syndrome in dogs and cats: 21 cases (2000-2007). J Am Vet Med Assoc 2010;236:548-553.

McIntyre RL, Levy JK, Roberts JF, et al: Developmental uterine anomalies in cats and dogs undergoing elective ovariohysterectomy. J Am Vet Med Assoc 2010;237:542-546.

Miller DM: Ovarian remnant syndrome in dogs and cats: 46 cases (1988-1992). J Vet Diagn Invest 1995;7:572-574.

Okkens AC, van de Gaag I, Biewenga WJ, et al: Urological complications following ovariohysterectomy in dogs [in Dutch]. Tijdschr Diergeneeskd 1981;106:1189-1198.

Poth T, Bruer W, Walter B, et al: Disorders of sex development in the dog-Adoption of a new nomenclature and reclassification of reported cases. Anim Reprod Sci 2010;121:197-207.

Schultz KS, Waldron DR, Smith MM: Inadvertent prostatectomy as a complication of cryptorchidectomy in four dogs. J Am Anim Hosp Assoc 1996;32:211-214.

Sontas BH, Gürbulak K, Ekici H: Ovarian remnant syndrome in the bitch; a literature review. Arch Med Vet 2007;39:99-104.

High quality, high volume sterilization programs

Brenda Griffin
Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL

Abstract

High quality, high volume spay neuter (HQHVSN) programs are efficient surgical initiatives designed to provide services to cats and dogs that would otherwise be unlikely to be neutered. Services typically target the pets of low-income owners and community cats with the goal of decreasing shelter impoundment and euthanasia. In the context of HQHVSN programs, the recommended timing for neutering dogs and cats is before adoption and before puberty. By concentrating on a single practice area, HQHVSN programs strive to improve surgical outcomes and reduce complications, while simultaneously reducing costs. Published veterinary medical guidelines for HQHVSN programs include recommendations for preoperative care, anesthetic management, surgery and post-operative care. These guidelines apply to a variety of program types including stationary and mobile services. Various program models facilitate the provision of services depending on the geographic needs and population. Formal mentorship programs and veterinary support facilitate program development. Programs should include an evaluation component so that the impact on cat and dog populations can be better assessed.

Keywords: Spay, neuter, castration, animal shelter, overpopulation, shelter medicine

Neutering is widely accepted as an essential component of preventive healthcare for cats and dogs in the United States[1] and represents the best method of birth control for these species. High volume, high quality spay-neuter programs are "efficient surgical initiatives that meet or exceed veterinary medical standards of care in providing accessible, targeted sterilization of large numbers of cats and dogs in order to reduce their overpopulation and subsequent euthanasia".[2] The purpose of this review is to describe the need for HQHVSN programs and to review the recommended veterinary medical guidelines and various models of these programs.

The need for high quality, high volume spay-neuter programs

Population estimates

According to the 2011-2012 American Pet Products Association National Pet Owners Survey, US households own approximately 86 million cats and 78 million dogs.[3] Current estimates indicate that between five and seven million cats and dogs enter US animal shelters each year, and approximately three to four million of them are euthanized.[4] Notably, cat impoundments and euthanasias significantly outnumber those of dogs in many animal shelters. In addition to the large numbers of cats that enter shelters, millions of others live in communities as unowned free-roaming strays with their reproduction frequently resulting in un-socialized feral cats. In fact, these "community cats" may produce up to 80% of the kittens born annually in the United States.[5] Thus, community cats represent both an effect of feline overpopulation as well as a significant source of it.

The population of unowned community cats present in a given locale is calculated by estimating one cat per six residents.[5] This formula is based on composites from multiple surveys.[5] A spreadsheet for calculating both the owned and unowned, outdoor cat population in a given community and its relationship to shelter impoundment is available at: http://www.sheltermedicine.com/documents/shelter-and-outdoor-cat-population-calculator.

Targeting

In order to maximize their impact, HQHVSN programs should target recognized sources of surplus cats and dogs. These include those cats and dogs that would otherwise be unlikely to be neutered, including both owned pet cats and dogs from low-income households and unowned community cats.

Cats and dogs in animal shelters frequently originate from low-income households,[6,7] thus HQHVSN programs strive to provide opportunities for affordable surgery for this population.

The traditional approach to controlling free-roaming, unowned community cats has been trapping and euthanasia by animal control agencies. However, large scale trap and kill programs, which would be necessary for even temporary population control, have not been widely implemented and even small scale attempts at trapping and euthanizing cats frequently result in public outcry. In contrast, the provision of affordable services to neuter free-roaming community cats raises awareness that cats require care and enables people to "do the right thing" when cats take up residence on their property or in their neighborhood.[8] These cats develop higher body condition scores following neutering,[9] and urine marking, fighting, breeding and roaming are dramatically reduced, making them less likely to be targeted as public nuisances.

Timing

Neuter before adoption

Surgical sterilization prior to release to adopters, including kittens and puppies as young as six weeks old, remains the most reliable and effective means of preventing unwanted reproduction of cats and dogs.[2,10] Approximately 20% of owned cats and dogs originate from animal shelters,[3] and many of these are adopted as young kittens and puppies. Animal shelters generally require that adopted animals be neutered, yet compliance rates for neutering following adoption average only 50-60%.[11] Neutering all cats and dogs prior to adoption ensures control of reproduction and sets an example of responsible ownership for the community. Neutering also improves the odds that pets will be retained in their homes because being sexually intact has been identified as the leading risk factor for owner relinquishment of cats and dogs.[12-14]

Neutering prior to puberty

To prevent pregnancy and avoid contributing to overpopulation, neutering should be performed before puberty. Given that queens may experience estrus as early as four to five months of age,[15] delaying spaying of juvenile cats is especially likely to result in a significant number of unintentional litters. Pet owner surveys reveal that many owned pets have one or more unintentional litters prior to being spayed.[16-18]

Currently, most private veterinary practices recommend six to nine months of age as the appropriate timing for neutering.[19] In contrast, professional guidelines for spay-neuter programs recommend that owned animals be scheduled for surgery at four months of age or older to allow time for the development of immunity through vaccination.[2] In the context of spay-neuter programs, the surgeon must always weigh the benefits of neutering when the opportunity arises against the risks posed by proceeding with surgery at the time of presentation. Often times, the presentation of a patient for surgery represents a one-time opportunity for that animal to be neutered, resulting in a risk:benefit ratio that generally favors surgery. This is true even for animals that are not yet vaccinated, have minor medical conditions, or that are pregnant or in heat.[2]

Recommendations for patients in private practice

It is important to recognize that the current standard age of six to nine months is not based not on a scientifically defined optimal age for neutering. This age was originally chosen because anesthetic and surgical techniques were less advanced at the time and surgical success was more likely in a larger patient. Despite considerable advances in anesthetic and surgical techniques and published data that illustrate shorter surgical times and lower complications rates for younger patients,[20] these recommendations have persisted.

Veterinarians routinely see kittens and puppies for a series of wellness exams and vaccinations between approximately six and 16 weeks of age. At the conclusion of the series, they typically advise owners to schedule an appointment in a few months for neutering. This gap in care likely contributes to

many pets being neutered following puberty and the births of many unintentional litters. In the author's opinion, most owned pets with private veterinarians are best served by neutering at five months of age following standard vaccinations. This allows time for development of immunity through vaccination while ensuring they are neutered prior to sexual maturity. Because there is no gap in veterinary care between the vaccine series and the surgical appointment, owner compliance may be improved since the owner establishes a routine of veterinary appointments for their cat during kitten- or puppy-hood visits.[21]

Health risks and benefits

There are risks and benefits to all medical and surgical procedures and it is the veterinary clinicians' responsibility to always weigh these in context. Detailed reviews of the risks and benefits of elective gonadectomy have been published.[19,22] The value of neutering as a preventive health care measure deserves emphasis. Most notably, when complete ovariohysterectomy or orchiectomy is performed, diseases of the uterus, ovaries, and testes, including cystic endometrial hyperplasia, pyometra, prostatitis and various cancers of the gonads themselves, are eliminated. Additionally, there are reports of significant reduction in the risk of mammary carcinoma in spayed versus intact females. Sexually dimorphic behaviors are also influenced by sterilization. Most notably, spraying in tomcats is generally eliminated and intermale aggression is frequently reduced in both cats and dogs.

Veterinary medical guidelines for spay-neuter programs

It is a common misperception that veterinarians performing a high volume of surgical sterilizations per day or those performing surgical procedures at a reduced cost are not providing quality care for their patients.[23-25] While exceptions may certainly exist, this is not typically the case. To the contrary, increased volume and reduced costs are not obtained by reducing quality. Concentrating on a single practice area has been used successfully in human surgery to significantly improve outcomes and reduce complications while simultaneously reducing costs. In HQHVSN programs, support teams, equipment, and protocols are geared towards safety, efficiency and humane quality care of large numbers of companion and feral cats and dogs. In pursuit of this effort, surgeons become extremely proficient at performing sterilization procedures and develop techniques unique to the field or utilize existing less well-known techniques that lead to increased efficiency.[21] Reported mortality rates in HQHVSN programs are very low. For example, the Humane Alliance Spay-Neuter Clinic in Asheville, NC reports a mortality rate of 0.03% (23,531 surgeries, 9 deaths) for 2012. This is lower than published mortality rates in small animal private practice and teaching hospitals[26-28] An example of postoperative complication rates is found in the table.

Professional guidelines for spay-neuter programs

State practice acts and professional organizations provide recommended guidelines for the practice of veterinary medicine, including spay-neuter surgery. Guidelines that specifically address spay-neuter practice have also been published and serve as valuable adjuncts to state and local practice acts.[2,29] Although specific protocols and procedures will necessarily differ among programs, certain aspects should remain consistent. A veterinarian should examine every patient and medical records should be prepared in compliance with state practice acts.[2] Systems for infectious disease control should be in place to prevent or minimize transmission among patients. Balanced anesthetic protocols are required, including the provision of adequate analgesia for all patients. Patients should be continuously monitored by trained hands-on observers, and emergency readiness plans should be in place.[2]

Regarding surgery, aseptic technique must never be compromised and separate sterile instruments should be used for all patients. Veterinarians (or veterinary students under their direct supervision) must perform all surgical procedures. For female patients, ventral midline, flank, and laparoscopic approaches are acceptable for ovariohysterectomy or ovariectomy. For males, a prescrotal or scrotal approach may be used for castration. Hemostasis must be ensured and verified prior to completion of any procedure. Either an interrupted or continuous suture pattern is acceptable for abdominal closure. The use of a permanent tattoo is recommended to mark cats and dogs at the time of spaying or neutering surgery

(figure 1). Removal of the tip of one of the ears (or pinna) is the accepted global standard for marking or identifying a neutered free-roaming or feral cat (figure 2).[2]

In the postoperative period, care should be taken to provide patients with a smooth recovery. Pet owners, caregivers or their agents should be provided with clear instructions for postoperative care. Finally, regular policies for managing complications and emergencies that occur within the 48-hour period after surgery must be in place.[2]

Model spay-neuter programs

A variety of programs have been designed and implemented to serve as efficient surgical initiatives providing accessible, targeted sterilization to large numbers of cats and dogs. These programs include stationary and mobile spay-neuter clinics, mobile army surgical hospital (MASH)-style operations, shelter services, feral cat programs and services provided through private practitioners.

In order for a community to support a stationary clinic, the National Spay Neuter Response Team of Humane Alliance (http://www.humanealliance.org) recommends a minimum human population of 250,000 within a 90-mile radius of a proposed clinic site. In order to be self-sufficient, these clinics typically must be capable of performing a minimum of 25 surgeries per day, five days per week, 48 weeks per year. Transport systems can be used to bring in patients from surrounding areas for surgery.[21]

Stationary clinics offer many advantages over mobile clinics, including greater daily surgical capacity compared to most mobile clinics, the ability to establish relationships with local veterinary practices and community members, and the possibility to hospitalize animals if necessary. Disadvantages include time and costs associated with establishing and maintaining a commercial facility and the potential for geographic limitation of the population in need of services. An alternative model of a stationary clinic that may counteract some of these disadvantages is the use of an existing veterinary hospital for regularly scheduled spay-neuter clinics. These "in clinic clinics" are especially valuable for serving the needs of targeted populations in rural communities.[21]

Mobile spay-neuter clinics often take one of two forms: MASH-style clinics and vehicles outfitted with surgical facilities. These models have the advantages of being able to target any geographic area in which services are needed and lower overhead costs. Disadvantages include limited animal housing and time constraints on spay-neuter efforts at a given location. Client communication and emergency care protocols must be especially well-planned as mobile clinics often leave an area after completing surgeries for the day, potentially leaving animals without the benefit of veterinary care shortly after recovery and release to their owners. In some states, practice acts prohibit or limit mobile neutering services.[21]

Optimizing success of HQHVSN programs

In order to optimize the impact of spay-neuter programs, the individual needs of a specific community must be identified. Factors to consider include both geographic and regional needs as well as socioeconomic and cultural barriers. A sterilization program should target the subpopulations of cats and dogs most responsible for influencing the population dynamics at a given locality. In other words, efforts should be focused on those cats and dogs that contribute the most to shelter impoundment and euthanasia and those that would not otherwise receive veterinary care.

Networking with other organizations and formal mentorship programs are useful for jumpstarting HQHVSN programs. The National Spay Neuter Response Team of Humane Alliance established a formal mentorship program in 2005. The program encompasses all aspects of clinic management and operations, from setting up a facility to training veterinary and support staff. Since its inception, the program has overseen the establishment of 118 clinics, which are collectively responsible for neutering more than 2.9 million cats and dogs since 2005.

Support from the local veterinary community also helps to ensure the success of spay-neuter programs. Historically, many veterinarians have opposed low cost spay-neuter services believing that they represent unfair competition for business. For this reason, individuals involved in spay-neuter

programs should communicate with local veterinarians about their goals in order to prevent or relieve misconceptions and facilitate collaboration and patient referral.[21]

Funding represents another key consideration for HQHVSN programs. As the single largest private funding agency for HQHVSN programs, Petsmart Charities has facilitated the development of many programs in the US. Developing a sound business plan is essential to ensure long-term sustainability. In some communities, public animal control funds are directed towards neutering pets. Both private humane organizations and private veterinarians have developed business models to serve targeted populations in need of low cost or subsidized spay-neuter services. Recruitment and training of veterinarians to staff HQHVSN clinics is another crucial consideration. The Association of Shelter Veterinarian's Veterinary Task Force to Advance Spay-Neuter was established in 2006 in order to improve the availability of resources and support for veterinarians in this practice area. Finally, collecting data to track the impact of spay-neuter programs on community cat population dynamics represents another important goal. The ability to demonstrate a measurable effect of spay-neuter efforts on shelter statistics serves to further legitimize programs and could be used to justify additional funding for them.[21]

Shelter euthanasia estimates have declined substantially over the past several decades, from an estimated 13.5 million cats and dogs in 1973 to current estimates of three to four million.[30] Some studies of community spay-neuter programs have demonstrated encouraging effects on shelter intake and euthanasia,[31] but more studies are needed to provide more detailed evidence on the effects of HQHVSN programs on animal populations. In conclusion, ensuring the availability of accessible, affordable HQHVSN programs provides opportunities to care for more cats and dogs, while helping people, promoting veterinary medicine, and providing humane alternatives to sheltering and euthanasia.

Acknowledgements

The author thanks Quita Mazzina and Karla Brestle of Humane Alliance.

References

1. AAHA-AVMA Preventive Healthcare Guidelines. Accessed 3-12-13 at: https://www.aahanet.org/PublicDocuments/AAHA-AVMA_PreventiveHealthcareGuidelines.pdf
2. Looney AL, Bohling MW, Bushby PA, et al: The Association of Shelter Veterinarians veterinary medical care guidelines for spay-neuter programs. J Am Vet Med Assoc 2008;233:74-86.
3. 2011-2012 American Pet Products Association:National pet owners survey. Accessed 3-12-13 at: http://www.americanpetproducts.org/press_industrytrends.asp.
4. ASPCA: Pet statistics. Accessed 3-12-13 at http://www.aspca.org/about-us/faq/pet-statistics.aspx.
5. Levy JK, Crawford, PC: Humane strategies for controlling feral cat populations. J Am Vet Med Assoc 2004;225:1354-1360.
6. Salman MD, New JC Jr, Scarlett JM, et al: Human and animal factors related to the relinquishment of dogs and cats in 12 selected animal shelters in the United States. J Appl Anim Welf Sci 1998;1:207-226.
7. Patronek GJ, Beck AM, Glickman LT: Dynamics of dog and cat populations in a community. J Am Vet Med Assoc 1997;210:637-642.
8. Griffin B: Care and control of community cats. In: Little S, editor. The cat: clinical medicine and management. St. Louis: Elsevier; 2012. p. 1290-1311.
9. Scott KC, Levy JK, Gorman SP, et al: Body condition score of feral cats and the effects of neutering. J Appl Anim Welf Sci 2002;5:203-213.
10. American Veterinary Medical Association: Policy statement on dog and cat population control. Accessed 3-12-2013 at: https://www.avma.org/KB/Policies/Pages/Dog-And-Cat-Population-Control.aspx.
11. Moulton C: Early spay/neuter: risks and benefits for shelters. American Humane Shoptalk 1990;7:1-6.
12. New JC, Salman MD, King M, et al: Characteristics of shelter-relinquished animals and their owners compared with animals and their owners in u.s. pet owning households. J Appl Anim Welf Sci2000;3:179-201.
13. Patronek GJ, Glickman LT, Beck AM, et al: Risk factors for relinquishment of cats to an animal shelter. J Am Vet Med Assoc 1996;209:582-588.
14. Scarlett JM, Salman MD, New JC Jr et al: Reasons for relinquishment of companion animals in U.S. animal shelters: selected health and personal issues. J Appl Anim Welf Sci 1999;2:41-57.
15. Griffin B: Prolific cats: the estrous cycle. Compend Contin Edu Pract Vet 2001;23:1049-1057.
16. Alexander SA, Shane SM: Characteristics of animals adopted from an animal control center whose owners complied with a spaying/neutering program. J Am Vet Med Assoc 1994;205:472-476.

17. Manning AM, Rowan AN: Companion animal demographics and sterilization status: results from a survey in four Massachusetts towns. Anthrozoos 1992;5:192-201.
18. Ipsos Marketing: Petsmart Charities, 2009 A and U barriers survey. Accessed 3-12-13 at: http://www.petsmartcharities.org/resources/resources-documents/PetSmartCharities_Research_AUBarriers.pdf.
19. Root Kustritz MV: Determining the optimal age for gonadectomy of dogs and cats. J Am Vet Med Assoc 2007;231:1665-1675.
20. Howe LM: Short-term results and complications of prepubertal gonadectomy in cats and dogs. J Am Vet Med Assoc 1997;211:57-62.
21. Griffin B, DiGangi B, Bohling M: A review of neutering cats. In: August JR, editor. Consultations in feline internal medicine VI. St. Louis: Elsevier; 2010. p. 776-792.
22. Reichler IM: Gonadectomy in cats and dogs: a review of the risks and benefits. Reprod Domest Anim 2009;44:29-35.
23. Woloshyn C: Cheap spays, neuters won't fix the animal overpopulation. Nov.2010. Accessed 3-12-2013 at: http://veterinarybusiness.dvm360.com/vetec/Veterinary+business/Cheap-spays-neuters-wont-fix-the-animal-overpopula/ArticleStandard/Article/detail/693827.
24. Adey R: Spay/neuter record attempt raises concerns. DVM Newsmagazine. March 2008. 36-37.
25. Robinson GW: How to keep a humane society hospital out of your community. Vet Econ 1990 June:32-41.
26. Pollari FL, Bonnett BN, Bamsey SC, et al: Postoperative complications of elective surgeries in dogs and cats determined by examining electronic and paper medical records. J Am Vet Med Assoc 1996;208:1882-1886.
27. Pollari FL Bonnett BN: Evaluation of postoperative complications following elective surgeries of dogs and cats in private practice using computerized records. Can Vet J 1996; 37: 672-678.
28. Brodbelt MA, Pfeiffer DU, Young LE, et al: Results of the confidential enquiry into perioperative small animal fatalities regarding risk factors for anesthetic-related death in dogs. J Am Vet Med Assoc 2008;233:1096-1104.
29. The Humane Society Veterinary Medical Association Rural Area Veterinary Services (HSVMA-RAVS): Fundamentals and standards of small animal field clinic surgery. Accessed 3-12-2013 at: http://www.ruralareavet.org/PDF/minimal_standards.pdf.
30. Scarlett JM: Pet population dynamics and animal shelters. In: Miller L, Zawistowski S, editors. Shelter medicine for veterinarians and staff. Ames (IA): Blackwell Publishing; 2004. p. 11-23.
31. White S C, Jefferson E, Levy JK: Impact of publicly sponsored neutering programs on animal population dynamics at animal shelters: the New Hampshire and Austin experiences. J Appl Anim Welf Sci 2010;13 191-212.

Figure 1: A linear tattoo along the ventral midline serves as an identifying mark to indicate this cat is neutered.

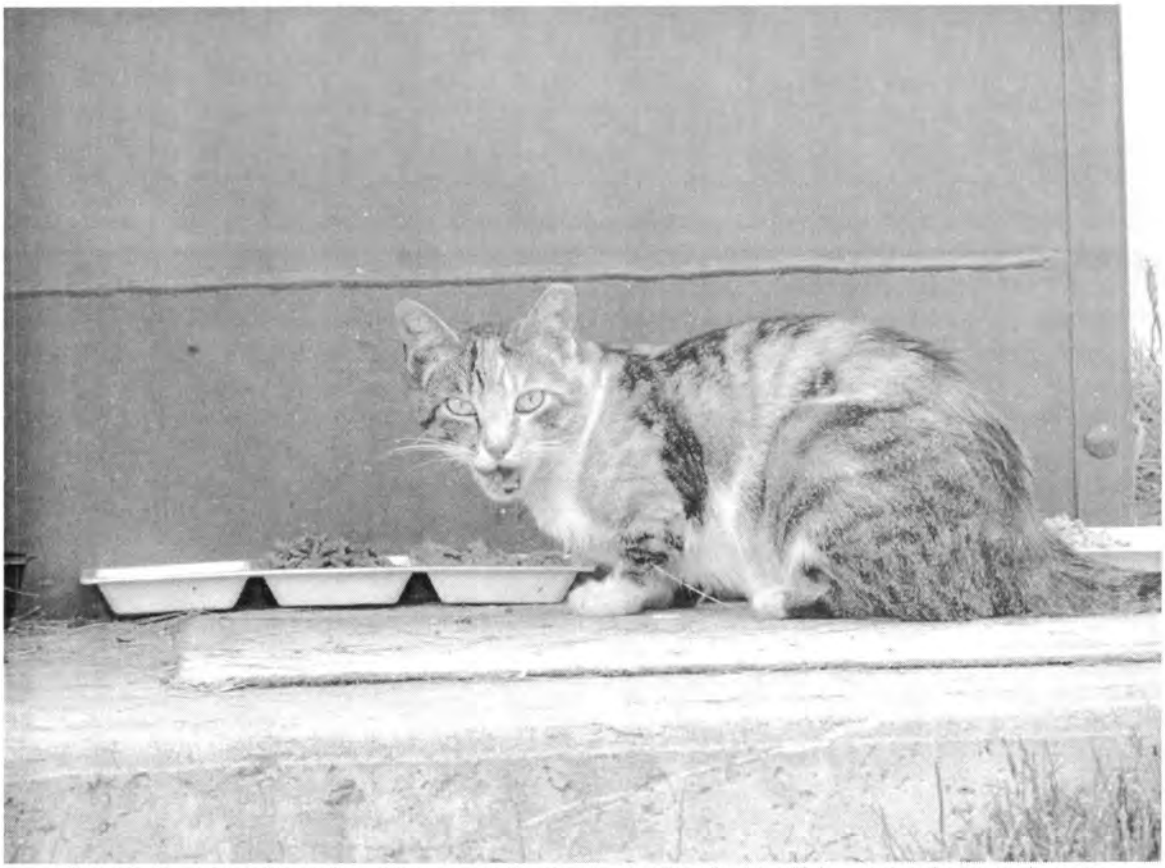


Figure 2: Cropping of the left ear tip serves as the standard method for identifying neutered community cats.

Table: Humane Alliance Spay Neuter Clinic 2012 post-operative complications

2012	Coughing, vomiting, diarrhea, lethargic, or other	114	0.48%
	Incision (noncompliant client)	8	0.03%
	Incision problem	176	0.75%
	Total rechecks	**298**	**1.27%**
	Normal	23,233	98.73%
	Total all	23,531	

Updates on anesthesia for non-obstetric and obstetric surgery during pregnancy

Maria Angeles Jimenez Lozano
Veterinary Medical Center, University of Minnesota, Saint Paul, MN

Abstract

Cesarean sections are a common procedure in veterinary medicine and specific anesthesia techniques have been recommended. Anesthesia for non-obstetric surgery during pregnancy is very rare. Independent of the purpose, anesthesia principles are going to be fairly similar although anesthesia for non-obstetric surgery includes special considerations like teratogenicity of compounds used, prevention of premature labor, and avoidance of fetal asphyxia. These concerns make anesthesia for the gravid animal a unique and problematic situation for the veterinarian. The main objective is to provide the safest possible anesthetic to the mother while preserving fetus safety. This review focuses on fundamental maternal physiological principles, core to the adequate care of pregnant mothers and fetuses, and reviews the most recent literature on the subject to provide tips and recommendations for the veterinarian whenever this challenging clinical scenario arises.

Keywords: Anesthesia, pregnancy, fetal safety, cesarean, non-obstetric

Introduction

Anesthetic management of pregnant patients represents a big challenge for the veterinarian. Due to uncertain drug effects on fetal development and high maternal anesthetic risk, any unnecessary anesthesia during pregnancy should be postponed. However on occasion anesthesia is required and the most sensible approach requires a good understanding of maternal and fetal physiology, altered drug pharmacology, and thorough risk analysis of the clinical situation. The most recent veterinary literature shows maternal mortalities of 9 to 16% in mares,[1-4] 19% in sheep and goats,[5] and 1% in pregnant dogs[6] presenting for cesarean section with or without dystocia. Although maternal mortality associated with cesarean section has decreased in recent years due to the introduction of improved anesthetic techniques and postoperative care, the risk associated with anesthesia for obstetric and non-obstetric procedures during pregnancy is still much higher in veterinary medicine than in human medicine.

If anesthesia is warranted for a pregnant patient, the main principles for the anesthetic management will be similar whether the surgery procedure is obstetric or not. They are summarized in the following points:

- Optimization and maintenance of normal maternal physiologic function
- Optimization and maintenance of utero-placental blood flow and oxygen delivery
- Avoidance of myometrium stimulation and use of known teratogenic drugs
- Avoidance of maternal stress
- Use of loco-regional anesthesia techniques as much as possible

Maternal safety: physiological adaptations to pregnancy

In general, pregnancy can be seen as a hypermetabolic state accompanied by many physiological changes to support that increased energy demand. Most of those changes happen under the influence of gestational hormones in order to ensure adequate supply of oxygen and nutrition to the fetus while other changes are a result of mechanical effect of the expanding gravid uterus. The following are the most clinically relevant changes from the anesthetic point of view.

Maternal cardiovascular changes

During pregnancy, the cardiovascular system undergoes substantial changes. Due to the rise in energy demand and oxygen requirements, the cardiovascular system is put under stress. The most relevant changes are a large plasma volume expansion, increased heart rate, and decreased overall systemic vascular resistance. All of these will increase cardiac output and maximize oxygen delivery to

more peripheral tissues like the uterus and placenta. In normal circumstances those changes will be compensated but in females already suffering from a cardiovascular disorder, pregnancy may worsen it and even put them into cardiac failure. There have been some reports in dogs and cats[7,8] with pregnancy-associated congestive heart failure, so any clinical signs should be investigated. If that is the case, the patient must be considered as very critical and should be treated for cardiac failure or at least stabilized as much as possible before anesthesia. Preservation of cardiac function and minimal cardiovascular depression should be priorities in determining the anesthetic protocol in these cases.

The decrease in systemic vascular resistance due to the pregnancy hormones prostaglandins and progesterone will affect directly the blood pressure and will predispose the mother to hypotension while anesthetized. As the uterus lacks an independent auto-regulatory blood flow mechanism and depends directly on maternal blood pressure, hypotension is a severe problem that needs to be addressed promptly when detected. In addition it has been seen that pregnant animals have a dampened baroreflex response and their ability to compensate for hypotension due to hypovolemia and hemorrhage is reduced; one study corroborates this, showing that pregnant dogs took longer to restore blood pressure in a model of acute hemorrhage compared to non-pregnant dogs[9]. Pregnant patients still preserved mechanisms of compensation like increase in heart rate, cortisol and vasopressin levels but to a lesser extent[9]. Accordingly, excessive bleeding during surgery can be very dangerous not only for the loss of ability to compensate but because the mother is already anemic, as described below.

Dilution anemia is another of the clinically relevant changes due to an enlarged plasma volume as water and sodium retention increases. If the pregnant patient suffers an excessive hemorrhage during surgery, ideally a blood transfusion or a hemoglobin-based oxygen carrying solution should be administered. Attempts to replenish volume with crystalloids or colloids may be only a temporary solution to support maternal hemodynamics and may be associated with dubious effects on oxygen delivery to the fetus, as they may produce further hemodilution. Moon et al in their study compared fetal oxygenation restoration by hetastarch versus blood or polymerized bovine hemoglobin solution in the ovine model of acute hemorrhage[10]. The results were that while all three products brought back maternal cardiovascular measurements to baseline, only blood or the hemoglobin-based solution could restore fetal oxygenation.

Another consideration for anesthesia during pregnancy is the position of the patient as it is associated with maternal hypotension. This is especially important during the last trimester since the gravid uterus will cause aorto-caval compression and decreased venous return whenever the patient is placed in dorsal recumbency. For that reason, the time the patient is in the dorsal position should be minimized by doing as much surgical preparation as possible while the animal is still awake and standing. Positioning the patient in a supine position with a left lateral tilt or oblique angle has been seen as a successful strategy to improve cardiovascular function in woman and most likely would be helpful for large animal species like equine as well. Nevertheless there are anatomical differences between species and a much greater collateral circulation which would explain why in veterinary species, positioning the pregnant patient on its back does not seem to be as problematic as in human medicine[11]. Veterinary research has shown that in full term pregnant anesthetized dogs, dorsal positioning did not cause more hypotension compared to lateral positioning or at a 10° to 15° oblique angle.[12,13] Even so, there is always potential for dramatic changes in hemodynamics caused by recumbency in anesthetized full-term pregnant animals; changing their positions slowly and thorough monitoring of cardiovascular parameters during and after positioning are recommended.

On the whole, maternal hypotension is very likely to occur under anesthesia and the treatment must be prompt and aggressive. Intravenous fluids, either colloids or crystalloids, can be very helpful to increase cardiac preload but inotrope or vasopressor agents may be more indicated especially when the patient is not hypovolemic. The use of the inotropes dobutamine and dopamine in gravid ewes has been studied; at high doses they both improved maternal cardiovascular parameters and increased the uterine vascular resistance.[14] Dobutamine induced uterine vasculature constriction to a lesser extent than dopamine. From this study it can be concluded that high doses of either should be avoided and dobutamine is a better treatment for hypotension than dopamine considering a selective preference for

beta adrenergic receptors and consequently better uterine blood flow. Traditionally ephedrine has been recommended for the treatment of hypotension in obstetrics. Ephedrine is a mixed alpha and beta adrenergic agonist drug. It may preserve utero-placental blood flow better than other vasopressors due to a superior beta adrenergic affinity and nitric oxide release that counteracts the vasoconstriction of alpha-1 receptors.[15] Nowadays, ephedrine use is controversial as some recent systematic reviews found more beneficial the use of other vasopressors like phenylephrine for the treatment of spinal anesthesia hypotension in parturient women. The results of those reviews were that the risk of fetal acidosis was decreased whenever phenylephrine was utilized[16] and that ephedrine was associated with lower umbilical cord pH.[17] Considering umbilical pH as an indirect measure of uterine blood flow, there is no support for the traditional recommendation of ephedrine treatment for spinal hypotension in parturient women. When it comes to veterinary species there is a lack of well-controlled studies performed on the subject and it is dangerous to extrapolate results and conclusions based on a human-specific model during labor. Recommendations to treat hypotension in pregnant anesthetized animals should be done cautiously and every case taken as an individual, keeping in mind the preservation of cardiac output, maintenance of adequate peripheral blood perfusion, and avoidance of excessive vasoconstriction that will increase arterial blood pressure but may compromise the fetus oxygenation.

Maternal respiratory changes

There are several important changes in the respiratory system during gestation. The pregnant patient is at a higher risk of hypoxia due to an increased metabolic oxygen demand that could be as high as 60%[18] increase at term, relative anemia, and important changes in lung volume. There is some compensation from the cardiovascular system by an increase in cardiac output, but some adjustments in the respiratory system must occur, too, to meet this higher oxygen demand and avoid hypoxemia. The respiratory rate and tidal volume increase, resulting in overall minute ventilation up to 45% to 70% higher than non-pregnant patients at full term.[18] This will increase oxygen to the required levels and will produce maternal hypocapnia (reduced carbon dioxide levels in blood) with slight respiratory alkalosis. That rise in maternal pH is limited by renal excretion of bicarbonate. Normal levels of arterial carbon dioxide ($PaCO_2$) in pregnant animals can be as low as 3.9-4.3Kpa (30-33mmHg).[19] It is necessary to keep $PaCO_2$ at those levels without any further hypocapnia as that would displace the maternal oxygen hemoglobin dissociation to the left increasing maternal hemoglobin oxygen affinity with a reduced oxygen delivery to the fetus. In addition, hypocapnia could cause uterine blood flow decrease by uterine vasculature constriction. On the other hand, hypoventilation and hypercapnia (abnormally high levels of carbon dioxide in blood) are not much better as the increased maternal $PaCO_2$ will limit the gradient for CO_2 diffusion from fetal to maternal blood leading to fetal acidosis. It is very important to keep adequate levels of oxygen and carbon dioxide; that is why tight control and monitoring of maternal ventilation is needed. Sometimes artificial ventilation will be mandatory, especially during the last trimester as the weight of the gravid uterus will worsen the typical hypoventilation caused by the respiratory depression and muscle relaxation due to the anesthetics in the recumbent anesthetized patient.

Other respiratory changes include a diminution in lung volumes, specifically the functional residual capacity (FRC), due to cranial displacement of the diaphragm with the expanding gravid uterus. This implies a reduction of residual volume in the lungs which gets near to the closing volume. That makes the alveoli prone to collapse, produces atelectasis, and reduces ventilation capacity and gas exchange efficiency. During anesthesia it might be very helpful to keep the patient on artificial ventilation with the application of periodic alveoli recruitment maneuvers and positive end expiratory pressure, in order to counteract hypoxemia and hypercapnia.

The pregnant patient is at most risk of hypoxemia during induction of anesthesia. At that stage, apnea may develop after administration of induction drugs. During apnea, gas exchange continues in the lungs thanks to the reserve gas volume FRC, but that reserve is smaller in the pregnant patient. That factor, combined with the raised oxygen demand, increases the risk of maternal hemoglobin desaturation and hypoxemia. Pre-oxygenation with 100% oxygen at 3-5 liters per minute via face mask or flow by, for

at least five minutes before induction of anesthesia, is the best way to prevent hypoxemia and is highly recommended.

Maternal neurological changes

Circulating gestational hormones have important effects on the anesthetic. On one side, progesterone is well known for its sedative effects.[20] On the other side, higher endorphin levels make pregnant patients more tolerant to surgical stimuli as their pain threshold is increased. These hormones are directly responsible for a decreased anesthetic requirement in pregnant patients, as well evidenced in the veterinary literature. Studies in pregnant sheep and women showed that the minimal alveolar inhalant concentration required to keep them at a surgical anesthesia plane is reduced by 25% for halothane and 40% for isoflurane compared to non-pregnant individuals.

The maternal neural tissue becomes more sensitive to local anesthetics so lower doses should be used in loco-regional anesthesia techniques like epidurals. It is important to remember too that the epidural space becomes smaller with pregnancy due to compression of the inferior venous cava by the gravid uterus, producing engorgement of the sinus venous plexus in the spine canal. As a consequence, lower dose-volumes for drugs should be used. Otherwise they could spread cranially too fast and too far, causing adverse effects like respiratory paralysis or central nervous system toxicity.

The autonomic system seems to have a parasympathetic predominance during the first trimester of pregnancy although this changes towards the last trimester when there is less vagal tone and sympathetic system is more prevalent. It would be important to minimize maternal stress as much as possible as this could trigger sympathetic system over-activity with tachycardia and increased peripheral vascular resistance, compromising uterine blood flow and putting the life of the fetus at risk.

Maternal physiological changes in other systems

Progesterone and estrogens decrease the lower esophageal sphincter tone. During pregnancy there is also an increase in intra-abdominal pressure, in addition to an increased gastric acidity and slower gastric emptying. The combination of these factors increases the risk of regurgitation for the pregnant patient under anesthesia. Regurgitation can have fatal consequences like aspiration pneumonia, esophagitis, and esophageal stricture. Although regurgitation in pregnant woman is a real concern, the evidence for its occurrence in veterinary species is scarce. In one prospective study[21] five out of nine pregnant dogs that died after cesarean section had evidence of pneumonia but no regurgitation or aspiration of gastric content could be documented. To date there are no veterinary studies looking specifically at increased risk of regurgitation under anesthesia during pregnancy. In any case, most anesthetists' recommendations are to secure and seal the airway with an endotracheal tube as quickly as possible after induction of anesthesia to avoid aspiration pneumonia. Another recommendation is to pre-treat the patients with pro-kinetics, H_2 receptor antagonists and/or proton pump inhibitors to increase gastrointestinal motility and make the gastric content less acidic.

Fetal safety

Avoidance of fetal asphyxia

Fetal asphyxia may occur as a consequence of maternal hypoxemia, decreased intrauterine flow, or by inefficient maternal oxygen transfer to the fetus. Maternal hypoxemia will cause utero-placental vasoconstriction and decreased perfusion, the fetus will become hypoxic and acidotic, and ultimately will die.

There is a very tight relationship between maternal and fetal $PaCO_2$. High levels of CO_2 in maternal blood will limit the gradient necessary for the gas exchange among uterine and umbilical vessels; therefore, the fetus will be unable to offload CO_2 and will become acidotic. Fetal acidosis causes myocardial depression and may progress to fetal death. On the other hand, maternal hypocapnia will cause uterine vasoconstriction and reduced fetal hemoglobin oxygen upload. These have already been discussed as part of maternal respiratory changes.

Close monitoring of the maternal respiratory system is essential for fetal safety. Pulse oximetry can be very useful for detecting hypoxemia and capnography can be used to monitor maternal levels of expired carbon dioxide; alternatively, arterial blood samples can be taken regularly to measure arterial blood gases. Measure should be taken to ensure normal levels of oxygen and carbon dioxide in the pregnant patient. In most cases, artificial ventilation is the best way to maximize oxygenation and ensure normocapnia. One of the downsides of the mechanical ventilation is the possible detrimental effect on the cardiovascular system. Special attention must be paid to positive pressures applied during ventilation when maternal hemodynamics are not stable, since positive pressure in the thoracic cavity will decrease venous return to the heart and worsen arterial blood pressure further.

Utero-placental circulation is not auto-regulated and depends directly on maternal blood pressure and cardiac output. Maternal cardiovascular parameters must be monitored and maintained at acceptable levels. As cardiac output measurement for clinical veterinary anesthesia is not a realistic option, we rely on arterial blood pressure monitoring complemented with capillary refill times, mucous membrane color, heart rate and even end tidal carbon dioxide to provide more information on blood flow and adequacy of peripheral tissue perfusion. Maintenance of satisfactory maternal cardiovascular parameters is crucial to avoid fetal asphyxia; maternal hypotension must be detected and aggressively treated. The use of balanced anesthetic techniques, close anesthesia depth monitoring and careful anesthetic titration to effect, together with intravenous fluids and the use of inotropes and vasopressors as needed, are some of the techniques we can use to counteract hypotension. The use of some specific inotrope-vasopressor drugs like ephedrine for obstetrics has already been discussed.

Teratogenicity and anesthesia

Teratogenicity is induction of any functional or anatomical abnormality in the newborn caused by a prenatal treatment. Virtually every drug has teratogenic potential in certain species, if administered in sufficient amount, for a sufficient length of time during a particular gestational period. Most drugs used for anesthesia have ideal properties like low molecular weight, low ionization and high liposolubility. Those characteristics allow them to rapidly cross the brain-blood barrier and at the same time cross the blood-placenta barrier. Consequently, the teratogenic potential of these drugs will depend on the stage of fetal development. During conception and implantation of the embryo, it is an "all or nothing" situation and either an abortion occurs or the embryo survives intact after the drug exposure. Later in the first trimester or period for organogenesis is when the fetus is more vulnerable to teratogen exposure. As the tissues differentiate, anatomical and structural malformations might be induced. Anesthesia and sedation during the first trimester should be avoided if possible. Functional abnormalities and fetal and organ growth retardation are associated with drug exposure during late pregnancy.

In experimental studies teratogenicity in laboratory species when exposed to specific drugs was demonstrated as follows:

- Nitrous oxide inhibits methionine synthetase activity interfering with DNA formation and myelin deposition. It has been shown that prolonged exposure of nitrous oxide in rat embryos during peak organogenesis is teratogenic.[22,23] It is important to remark that these studies are based on a single species exposed to nitrous oxide for at least 24 hours, an unusual situation that would never be seen in clinical practice. Therefore, extrapolation of the study results and clinical application are questionable. There is no proof of teratogenicity in other species or after short time exposures.
- Inhalants have been studied under laboratory conditions in pregnant mice with conflicting results. Halothane has shown teratogenicity when mice were exposed for more than 12 hours but other investigators could not corroborate those results under similar circumstances.[24] Others have studied isoflurane and enflurane exposure in pregnant mice and shown an increased incidence of cleft palate without any other abnormalities,[24] but again other studies comparing halothane, isoflurane, enflurane and a known teratogen did not show any major abnormality within the groups exposed to anesthetics.[25] Sevoflurane and desflurane are considered the safest as they have not shown any teratogen effect in animal studies.[24]

- Chronic use of diazepam during pregnancy has been associated with cleft lip and palate in human neonates, but the latest studies have failed to validate these results.[24] In animal studies, benzodiazepines have been associated with that malformation too, although the real danger of their use is unknown in different animal species as is the risk of a single does for a single anesthetic or sedation episode.
- Non-steroidal anti-inflammatory drugs (NSAIDs) should not be administered to pregnant animals. The teratogenic effect of these analgesic drugs when administered to the mother can be quite profound. These drugs inhibit cyclooxygenase (COX) production and decrease prostaglandin production; prostaglandins maintain patency of the ductus arteriosus and regulate pulmonary vasculature in the fetus. The use of NSAIDs could cause constriction or closure of the ductus and cause fetal pulmonary hypertension.[26,27] Oro-facial clefts in the fetus have also been associated with NSAID administration.[28] Fetal circulation disruption and even cessation of labor are other possible effects of these drugs because of the blockade of prostaglandins.[26] More recent studies[28] have shown an important role of COX-2 in fetal kidney maturation, so potential placental transfer of NSAID's from the mother can stop nephrogenesis in the fetus. Non-steroidal anti-inflammatory drugs should be withheld in the pregnant veterinary patient until scientific studies can prove their safety.

Whenever possible, elective surgery should be delayed at least until after the first trimester to minimize the risk of fetal demise or malformations due to teratogenicity. Ideally the anesthesia should be delayed until after term.

Prevention of pre-term labor/abortion

Anesthesia and surgery during pregnancy increases the risk of spontaneous abortion or pre-term labor, especially if the surgery involves intra-abdominal procedures. When those are planned for the late pregnant patient, pre-term labor represents one of the main concerns for the anesthetist. The risk can be decreased by minimizing uterine manipulation and avoidance of anesthetic drugs that increase uterine tone or induce uterine muscle contraction.

The perioperative use of prophylactic tocolytic therapy in the pregnant animal to prevent premature labor or abortion has not been studied; in humans that technique has shown to be controversial due to the maternal side effects and uncertain efficacy during non-obstetric surgery. There is a published case report in which the tocolytic drug isoxsuprine was used as part of the perioperative anesthetic protocol in a late-term gravid cow undergoing general anesthesia for a metacarpal fracture repair.[29] In this case the outcome was positive and not only did the cow recover well from surgery but a healthy calf was born at term. Although it is not possible to prove that the tocolytic helped in the successful outcome of the case, it is true that the anesthetic protocol contained two drugs known for causing increased uterine tone and decreased uterine blood flow. Those drugs are ketamine and xylazine which should be avoided as much as possible in the late pregnant patient. If there are no other choices, as in the case report, then a tocolytic might help to relax the uterus and counteract uterine muscle contractions.

Xylazine is an alpha-2 agonist sedative drug widely use in veterinary medicine. Alpha-2 agonists in general have potent side effects like reduction of cardiac output, which will impair fetal oxygen delivery. For that reason, these drugs should be used very cautiously for the pregnant patient or not used at all if possible. Additionally, they can increase uterine motility. Xylazine use has been related to early parturition when administered during the third trimester.[30]

Ketamine is a dissociative anesthetic injectable agent used in all veterinary species. Its use is a bit controversial in obstetrics; on one hand, it has very favorable cardiovascular properties helping to maintain the maternal hemodynamics but on the other hand, there is an increase in uterine tone and it might cause uterine vasoconstriction. The safest way to use ketamine would be as part of a balanced anesthetic technique where other anesthetic drugs can counteract the negative effects of ketamine by providing muscle relaxation and some vasodilation to help uterine perfusion. Moreover, ketamine can be administered at small bolus doses and in small dose constant rate infusions so side-effects like tachycardia and hypertension are less likely to occur.

Maternal stress can cause spontaneous abortion and it is considered one of the major risks during the first trimester. In humans, there is clear association with increased maternal cortisol as a physiological measure for stress and spontaneous abortion during the first three weeks.[24] It is very important to plan well for the procedure so stress on the mother is minimal. It is true that some drugs can jeopardize the life of the fetus and some clinicians attempt to perform minor procedures by administering light sedation to the mother. However, if the mother gets quite stressed that can be very detrimental for the fetus too. Sometimes the risks for sedating pregnant patients before anesthesia must be weighed against preparing the patient without sedatives and dealing with maternal stress. Emergency cesarean section would be one of those occasions where drug effects on the fetus could be more important to consider than maternal stress. Fetal life is already in danger and the fetus will have more chance of survival if it gets delivered with the least amount of anesthetic possible so it suffers less cardio-respiratory and neurological depression and rapidly becomes an independent newborn, vigorous and strong enough to nurse straight away.

Putting everything together: general anesthetic recommendations

The best recommendation is to use a balanced anesthetic technique, where multiple drugs induce anesthesia targeting unconsciousness, muscle relaxation, immobilization and analgesia.

Pharmacological considerations

Pharmacokinetics and pharmacodynamics are altered during pregnancy so careful titration of drugs is recommended. Moreover the anesthetic and analgesic maternal requirements are decreased due to the effect of gestational hormones on the central nervous system.

The increase in blood volume causes a physiological hypo-albuminemia that alters drug protein binding and leaves a relative higher fraction of unbound/free drug. That potentiates the drug effect and puts the patient at risk of overdose, especially when drugs that readily bind protein are used.

Most of the anesthetic drugs are not highly ionized and very lipophilic so they can readily cross the blood-brain barrier; unfortunately, they cross the placenta barrier with the same facility. The amount that gets transferred depends on the placental blood flow and maternal protein binding, while the amount that is available for the fetus will depend on fetal uptake, metabolism, and clearance. In humans, it is known that the fetal liver is active and able to perform some drug metabolism, whereas in dogs this is not the case at all and the fetus depends entirely on maternal circulation, metabolism, and elimination of drugs and metabolites. It is safe to assume that drugs will affect the dam and fetus similarly. Drugs with low ionization, like the opioids and local anesthetics that are weak bases, will transfer to the fetus under a gradient concentration until a maternal-to-fetal equilibrium is reached. If for any reason the fetus becomes acidotic, these weak bases tend to get ionized and trapped in the fetal circulation. Once ionized, those molecules cannot cross the placenta back to the maternal circulation. This well-known phenomenon, ion entrapment, will cause accumulation of drug in the fetal tissues.

Premedication

One of the main uses of premedication is to minimize patient stress and that is very important in the pregnant patient. The clinician might consider not sedating the patient if it is calm or is very depressed and sick. Otherwise, mild sedation with narcotics can be very beneficial and helps to decrease required doses of induction drugs. Opioids have been shown to be the safest analgesic drugs for mother and fetus. They should always be part of the anesthetic protocol as they are potent analgesics, provide sedation, and have great sparing effects on induction and maintenance anesthetic drugs. Their side effects are minimal and dose-dependent and most of them can be easily reversed by the antagonist naloxone. Opioids should be carefully chosen based on the circumstances of the case. For cesarean sections an opioid that has short duration and is easy to antagonize should be chosen to improve the neonates' chances for survival.

The use of sedatives like alpha-2 agonists is not recommended due to their potent side-effects on the cardiovascular system and uterus. If the patient is very aggressive and represents a danger to itself or

to the veterinary team alpha-2s could be chosen as they are the best sedatives available. Detomidine for large animals and dexmedetomidine for small animals are a safer option for the fetus than xylazine but still the use of these drugs is a high risk as they can cause fetal asphyxia or premature labor. On a more positive note, alpha-2s are easily reversed. If they must be used , the best option would be to reverse them with atipamezole once the sedation is not needed any longer.

Acepromazine is a major tranquilizer heavily used in animals as part of the anesthesia or just for sedation. It produces a calming effect and decreases anxiety. The negative side is that it causes vasodilation and can potentiate maternal hypotension under anesthesia. Because this tranquilizer has a very long duration of action and has no antagonist, it is better not to use it for the pregnant patient if possible. It is not recommended for cesarean section.

Other tranquilizers like benzodiazepines can potentiate sedative effects of opioids and provide muscle relaxation with very minimal side effects and a wide safety margin. Their effects disappear with administration of the antagonist flumazenil. The only concern about this class of drugs is the potential for teratogenicity. The evidence is scarce and inconsistent and the references are vague, therefore the use of benzodiazepines as part of anesthesia during pregnancy is probably a low risk worth taking. The benefits of including these drugs in the anesthetic protocol outweigh the low risk of teratogenicity, especially if they are used at clinical doses during a single anesthetic episode, rather than a prolonged chronic maternal use. Whenever benzodiazepines are used in cesarean section, the antagonist flumazenil must be available for administration to the newborns so they are not too sedated to nurse and even to hypoventilate due to the muscle relaxation.

Induction of anesthesia

There is no standardized technique for anesthesia induction in the pregnant patient. Two min techniques, either inhalants or injectable, can be employed. Both of them have advantages and disadvantages.

The main focus during induction is speed. The anesthetist must be able to gain control of the patient's airway as soon as possible, as the predisposition to hypoxemia and high risk of regurgitation are well known. It is essential to secure and seal the airway with an endotracheal tube to avoid aspiration of regurgitation, and to start the delivery of oxygen at 100% to counteract hypoxemia. Maternal ventilation can be gently supported if the induction drugs produce hypoventilation or apnea. Injectable anesthetic agents will provide the quickest induction, which is why the author prefers this technique over the use of inhalants. Probably the best injectable anesthetics are propofol and etomidate. Propofol provides a smooth, quick induction although it causes profound cardiovascular depression by producing hypotension after excessive vasodilation. This cardiorespiratory depression is dose-dependent and lasts a few minutes due to propofol's rapid re-distribution; consequently, most pregnant patients tolerate it with no significant problems. Etomidate is a higher profile induction agent, much safer than propofol, with minimal effect on the cardiovascular system so it is advantageous if the mother's health is critical or if she suffers from a cardiac disorder. On the other hand, etomidate induction is not as smooth as propofol and it is much more expensive. Both agents have been safely used in cesarean sections in small animals with high rates of neonatal survival and increased newborn vigor. In contrast, thiopental, thiamylal, and ketamine, when used as induction agents for dog cesarean section, have been associated with decreased puppy vigor.[31] Other studies done in puppies born by cesarean support those results and show that puppies with the highest neurological depression were the ones born from dams anesthetized with ketamine, followed by the ones with thiopental and then by the ones with propofol.[32]

Inhalant induction is another possibility and probably the most advantageous for the fetus about to be delivered by cesarean section due to the rapid elimination in the lungs while breathing with almost no metabolism or accumulation. Due to the cardiorespiratory changes during pregnancy, inhalant induction via face mask or induction chamber is much quicker than in the normal patient, nevertheless this induction is still very slow compared to injectable drugs and is rarely smooth. The main risk factors to be considered for an inhalant induction are: slow and long induction, increased maternal stress, and the profound cardiovascular effects of the inhalants as they have to reach very high levels to produce

unconsciousness. If this is the preferred induction method, volatile agents that are the least pungent and have the best pharmacokinetic properties should be employed. At this time, the best agent available to induce anesthesia is sevoflurane. Isoflurane is more soluble than sevoflurane so the induction would take longer. Desflurane is less soluble than sevoflurane and induction would be quicker but the odor is very pungent and will be less acceptable to the patient, prolonging the induction and making it more stressful.

Anesthesia maintenance

Most volatile agents used nowadays in veterinary medicine are very safe for mother and fetus. Isoflurane, enflurane and halothane have shown teratogenicity in specific research performed with rat embryos but other research studies fail to reproduce those results and it was concluded that they were all safe. As the evidence is so inconsistent, those inhalants should be used for clinical anesthesia of the pregnant patient as they represent low teratogen risk if any at all. Neither sevoflurane or desflurane have been associated with teratogenicity[24] so they are the inhalant of choice during pregnancy.

In contrast, nitrous oxide is a known teratogen therefore its use is not recommended in pregnant patients. It is commonly used for pain relief during labor in human medicine but it might exacerbate hypoxemia in the newborn due to diffusion hypoxia. This could happen in neonates during cesarean section so the author cannot recommend its use for cesarean because neonatal resuscitation is already very challenging.

There are studies using injectable techniques to maintain anesthesia for non-obstetric surgical procedures in pregnant pony mares, which concluded that intravenous anesthesia provides safer anesthesia than halothane with fewer cardiovascular depressant effects;[33] data comparing other inhalant agents[33] are not available. One of the studies used propofol as total intravenous anesthesia and demonstrated that propofol anesthesia was smooth with satisfactory cardiovascular function in the mare and fetus; the authors concluded that propofol is a suitable safe option for pregnant ponies.[34] The other study involved the same species and very similar scenario but with a different total intravenous technique which consisted of a combination of ketamine, guaifenesin and detomidine; the results were equally satisfactory for both mare and fetus so the authors recommended this protocol as a good technique for anesthesia in pregnant equidae.[33] The latter study seems to confirm that the use of detomidine is safer than xylazine if an alpha-2 agonist must be used, and that ketamine can be used as part of a balanced anesthetic protocol similar to the pregnant cow case report already discussed.[29]

Although there is not that much information about injectable techniques for anesthesia maintenance in pregnant small animals, Moon et al[31] concluded in retrospective studies that the use of propofol for induction and isoflurane for maintenance had a positive effect in vigor of puppies delivered by cesarean whereas the use of methoxyflurane and xylazine was associated with puppies born dead and the use of ketamine or barbiturates had a negative influence in puppy vigor.[31]

Local anesthesia

Spinal anesthesia presents the least placental drug transfer for the degree of anesthesia received.[24] This is especially important for the parturient patient undergoing cesarean for delivery because the less drug transferred to the fetus before delivery, the better the chances for the fetus to survive. In the prospective study by Luna et al[32] puppies delivered by cesarean from sedated mothers that received local anesthesia by epidural injection were livelier and had higher respiratory rates than pups delivered from dams that got general anesthesia with three different protocols.

Epidural anesthesia is probably the best loco-regional technique for the pregnant patient. It is indicated for any orthopedic or soft tissue procedure involving hind limbs, abdomen, genito-urinary and perineal region. It is a fairly easy technique with outstanding analgesia and great anesthetic sparing effects. Owing to those sparing effects, both mother and fetus are exposed to a lower amount of drugs with less potential for fetal toxicity, teratogenicity and better maternal cardiorespiratory function, reducing the risk for fetal asphyxia and death. Two things must be kept in mind when epidurals are performed in the pregnant patient: they can be more challenging than in the normal patient because the epidural space is reduced so local anesthetics doses should be reduced accordingly to avoid an excessive

cranial spread, and an epidural with local anesthesia might cause maternal hypotension due to sympathetic blockade. If that is the case it is important to recognize and aggressively counteract the hypotension as discussed above.

Any other loco-regional technique appropriate for the surgical procedure will be of benefit to the pregnant patient. When successful they reduce the overall anesthetic requirements and will provide stability to the anesthetic by blocking the sympathetic response to surgical stimulation. It is definitely worthwhile to administer local blocks, especially those that are familiar to the clinician. The supplies and drugs needed for local anesthetics are inexpensive and most times the morbidity associated with nerve blocks is very low. Clinicians must remember to reduce doses of local anesthetics to avoid toxicity as pregnant patients have been shown to have an increased pain threshold and a special sensitivity to local anesthetics in the neural tissue.

References

1. Byron CR, Embertson RM, Bernard WV, et al: Dystocia in a referral hospital setting: approach and results. Equine Vet J 2003;35:82-85.
2. Maaskant A, DeBruijn CM, Schutrups AH, et al: Dystocia in Friesian mares: prevalence, causes and outcome following caesarean section. Equine Vet Educ 2010;22:190-195.
3. Abernathy-Young KK, LeBlanc MM, Embertson RM, et al: Survival rates of mares and foals and postoperative complications and fertility of mares after cesarean section: 95 cases (1986-2000). J Am Vet Med Assoc 2012;241:927-934.
4. Arnold CE, Payne M, Thompson JA, et al: Periparturient hemorrhage in mares: 73 cases (1998-2005) J Am Vet Med Assoc. 2008;232:1345-1351.
5. Brounts SH, Hawkins JF, Baird AN, et al: Outcome and subsequent fertility of sheep and goats undergoing cesarean section because of dystocia: 110 cases (1981-2001). J Am Vet Med Assoc 2004;224:275-279.
6. Moon PF, Erb HN, Gleed RD, et al: Perioperative risk factors for puppies delivered by cesarean section in the United States and Canada. J Am Anim Hosp Assoc 2000;36:359-368.
7. Stoneham AE, Graham J, Rozanski EA, et al: Pregnancy-associated congestive heart failure in a cat. J Am Anim Hosp Assoc 2006;42:457-461.
8. Sanduski GE, Cho DY: Congestive heart failure in a dog associated with pregnancy. Cornell Vet 1984;74:60-64.
9. Brooks VL, Keil LC: Hemorrhage decreases arterial pressure sooner in pregnant compared to nonpregnant dogs: role of baroreflex. Am J Physiol Heart Circ Physiol 1994;266:H1610-H1619.
10. Moon PF, Bliss ST, Posner LP, et al: Fetal oxygen content is restored after maternal hemorrhage and fluid replacement with polymerized bovine hemoglobin but not with hetastarch in pregnant sheep. Anest Analg 2001;93:142-150.
11. Pascoe PJ, Moon PF: Periparturient and neonatal anesthesia. Vet Clin North Am Small Anim Pract 2001; 315-340.
12. Probst CW, Webb AI: Postural influence on systemic blood pressure, gas exchange and acid/base status in the term-pregnant bitch during general anesthesia. Am J Vet Res 1983;44: 1963-1965.
13. Probst CW, Broadstone RV, Evans AT: Postural influences on systemic blood pressure in large full-term pregnant bitches during general anesthesia. Vet Surg 1987;16:471-473.
14. Fishburne JI, Meis PJ, Urban RB, et al: Vascular and uterine responses to dobutamine and dopamine in the gravid ewe. Am J Obstet Gynecol 1980;137:944-952.
15. Li P, Tong C, Eisenach JC: Pregnancy and ephedrine increases the release of nitric oxide in ovine uterine arteries. Anesth Analg 1996; 82:288-293.
16. Lee A, Ngan-Kee WD, Gin T: A quantitative, systematic review of randomized controlled trials of ephedrine versus phenylephrine for the management of hypotension during spinal anesthesia for cesarean delivery. Anesth Analg 2002;94:920-926.
17. Veeser M, Hofmann T, Roth R, et al: Vasopressors for the management of hypotension after spinal anesthesia for elective caesarean section. Systematic review and cumulative meta-analysis. Acta Anaesthesiol Scand 2012;56:810-816.
18. Mhuireachtaigh RN, O'Gorman DA: Anesthesia in pregnant patients for nonobstetric surgery. J Clin Anesth 2006;18:60-66.
19. Greene SA: Anesthetic considerations for surgery of the reproductive system. Semin Vet Med Surg (Small Anim) 1995;10:2-7.
20. Camann WR, Ostheitmer GW: Phsyiological adaptations during pregnancy. Int Anesthesiol Clin 1990;28:2-10.
21. Moon PF, Erb HN, Ludders JW, et al: Perioperative management and mortality rates of dogs undergoing cesarean section in the United States and Canada. J Am Vet Med Assoc 1998;213:365-369.
22. Fujinaga M, Baden JM, Mazze RI: Susceptible period of nitrous oxide teratogenicity in Sprague-Dawley rats. Teratology 1989;40:439-444.
23. Fujinaga M, Baden JM, Yhap EO, et al: Reproductive and teratogenic effects of nitrous oxide, isoflurane, and their combination in Sprague-Dawley rats. Anesthesiology 1987;67:960-964.

24. Allaert SEG, Carlier SPK, Weyne LPG, et al: First trimester anesthesia exposure and fetal outcome. A review. Acta Anaesth Belg 2007;58:119-123.
25. Mazze RI, Fujinaga M, Rice SA, et al: Reproductive and teratogenic effects of nitrous oxide, halothane, isoflurane, and enflurane in Sprague-Dawley rats. Anesthesiology 1986;64:339-344.
26. Mathews KA: Pain management for the pregnant, lactating and neonatal to pediatric dog and cat. Vet Clin North Am Small Anim Pract 2008;38:1291-1308.
27. Van der Veyver IB, Moise KJ: Prostaglandins synthetase inhibitors in pregnancy. Obset Gynecol Surv 1993;48:493-502.
28. Ericson A, Kallen BAJ: Nonsteroidal anti-inflammatory drugs during early pregnancy. Reprod Toxicol 2001;15:371-375.
29. Martin Jurado O, Jud R, Muggli E, et al: Balanced anaesthesia approach in a late-term gravid cow undergoing metacarpal fracture repair. Case reports in veterinary medicine. Volume 2011; Article ID: 743827. doi:10.1155/2011/743827.
30. Dehghani S, Behbodikhah A, Khorsand N: Clinical, haematological and biochemical effects of xylazine, ketamine and their combination in cattle and sheep. Vet Anaes Analg 1991;18 Suppl 1:S123-S128.
31. Moon-Massat PF, Erb HN: Perioperative factors associated with puppy vigor after delivery by cesarean section. J Am Anim Hosp Assoc 2002;38:90-96.
32. Luna SPL, Cassu RN, Castro GB, et al: Effects of four anesthetic protocols on the neurological and cardiorespiratory variables of puppies born by cesarean section. Vet Rec 2004;154:387-389.
33. Taylor PM, Luna SPL, White KL, et al: Intravenous anaesthesia using detomidine, ketamine and guaiphenesin for laparotomy in pregnant pony mares. Vet Anaes Analg 2001;28:119-125.
34. Taylor PM, Fowden AL, Sear JW: Propofol anaesthesia for surgery in late gestation pony mares. Vet Anaes Analg 2001;28:177-187.

Transitional cell carcinoma of the canine reproductive tract

Sara Fritz
College of Veterinary Medicine, University of Minnesota, St. Paul, MN

Overview

Transitional cell carcinoma (TCC) is the most common malignancy of the urinary tract in dogs and accounts for approximately 2% of all malignancies in dogs.[1] In a series of 102 dogs with TCC of the bladder, in 56% of the dogs the urethra was also involved and in 29% of dogs the prostate was involved.[2] Most TCCs are intermediate to high grade papillary infiltrative tumors that can be associated with a variety of clinical signs.[1] The etiology of canine transitional cell carcinoma is multifactorial. Risks factors include exposure to lawn chemicals, obesity, female sex and breed.[2] It is important to distinguish non-TCC conditions from TCC of the reproductive tract because the treatment and prognosis differ considerably.

Keywords: Transitional cell carcinoma, prostate, ductus deferens, vagina, vulva

Prostatic transitional cell carcinomas

Prostatic tumors are relatively uncommon in dogs, with a prevalence of less than 1%.[2] Despite this low incidence, the dog is one of the few domestic species to develop spontaneous prostate cancer, thus sparking interest in the dog as a comparative model for prostate cancer in men.[3] Human prostatic carcinomas arise almost exclusively in the peripheral zone, with fewer found in the transition and central zones.[3] It is hypothesized that prostatic cancer in men arises from acini in the peripheral or transitional zones previously affected with inflammatory prostatitis.[3] The surrounding prostatic epithelial cells undergo a morphological change secondary to this inflammatory insult. The morphological change is known as proliferative inflammatory atrophy (PIA).[3] In dogs, the precise location of origin of the carcinomas is unclear but there is growing evidence that these neoplasms may stem from ductular epithelium adjacent to the periurethral zone.[3] Much of the uncertainty regarding the site of origin of these neoplasms is due to the extensive co-involvement of the prostate gland and urethra that is present in most dogs with prostate cancer.[2]

The early stages of human prostate cancer are characterized by the histological pattern known as prostatic intra-epithelial neoplasia (PIN).[3] Morphologically, PIN is characterized as an intra-luminal proliferation of epithelium exhibiting varying degrees of malignant criteria. Prostatic intra-epithelial neoplasia is considered a precursor of human prostate carcinoma and occurs under the influence of androgenic stimulation in those patients at risk for prostatic carcinoma.[3] Prostatic intra-epithelial neoplasia cells are often found in close proximity to foci of PIN and prostatic carcinomas and commonly contain somatic mutations and possess an increased rate of cell division.[3] While histological lesions similar to PIN have occasionally been identified in canine prostate glands, the value of PIN as a cancer marker in dogs is unclear.[2] Although PIN has been detected in dogs with existing prostatic carcinoma, it has also been detected in dogs without evidence of prostatic disease, making its role in the dog less clear.[2] The occurrence of high grade PIN in dogs with concurrent carcinoma has been reported to range from 30-72%.[3] Histologically, most canine prostatic carcinomas are of an intra-alveolar pattern but many also contain similar patterns to TCC.

Overall, canine prostate carcinomas are malignant epithelial neoplasms that often arise from an urothelial or ductular origin rather than acinar because most canine tumors are androgen independent.[3] This differs from humans because most prostatic carcinomas in men are highly androgen dependent. Most tumors of the canine prostate are carcinomas and the majority are adenocarcinomas.[2] In a report of 14 dogs with prostate cancer, ten were diagnosed by histopathology as adenocarcinoma and four were undifferentiated carcinomas.[4] Other tumor types that have been identified in the prostate include TCC, fibrosarcoma, leiomyosarcomas and hemangiosarcoma.[4] Transitional cell carcinoma of the prostatic urethra frequently will invade the prostate and it may be difficult to distinguish primary TCC from secondary invasion of a urethral tumor.[5]

Elderly dogs are more commonly diagnosed with prostatic TCC, with a median age at diagnosis of 10 years.[2] Both intact and castrated dogs develop prostatic TCC although multiple studies have suggested there is an increased risk of prostatic carcinomas in castrated dogs compared to intact dogs with an odds ratio of approximately 2.3:4.3.[3] The Shetland sheepdog and Scottish terrier are at increased risk of developing prostatic TCC.[2] In a case control study in Scottish terriers, exposure to lawn herbicides and pesticides was compared between Scottish terriers with TCC and a control group. Transitional cell carcinoma risk was significantly higher in Scottish terriers that had been exposed to lawn herbicides and pesticides than in the Scottish terriers that were not exposed.[2] The "inert" ingredients of the lawn chemicals were speculated to be the probable carcinogens.[2] In the same study, dogs that ate vegetables at least three times a week along with their normal diet had a reduced risk of TCC.[2] Carrots given as treats were the most frequently fed vegetable in the study.[2] The female: male ratio of dogs with TCC has been reported to range from 1.7:1 to 2:1.[2]

Most canine prostatic TCCs are characterized by local invasion with a high propensity for regional and distant metastasis, and most dogs are diagnosed with advanced disease. The most common sites of metastasis are the regional lymph nodes, urinary bladder and lungs.[2] Similar to high grade prostatic carcinomas in men, canine prostatic TCC has a tendency to metastasize to bone, predominately to the lumbar vertebrae and pelvis. In one study, 42% of dogs had evidence of bone metastasis.[2] Younger dogs have been shown to be at an increased risk for metastasis to bone.[2]

Clinical signs in dogs with prostate TCC are variable and may be reflective of local and/or metastatic disease. Common clinical signs may include urinary tract signs (hematuria or stranguria), difficult or abnormal defecation (tenesmus or passage of flattened, ribbonlike stools), and a range of systemic signs of illness (lethargy, weight loss or inappetence) or lameness, pain and neurological deficits secondary to bone metastasis.[5] Dogs may present with a history of clinical signs that partially improve with empiric therapy and then return once the therapy is discontinued. In a retrospective study of 76 dogs with prostatic carcinoma, clinical signs were referable to the urinary tract in 62%, tenesmus was noted in 30%, signs of skeletal involvement were seen in 36% and signs of systemic disease occurred in 42% of the dogs.[5] If complete obstruction of urinary outflow results from prostatic compression or direct tumor extension into the urethra, hydroureter, hydronephrosis and renal failure may occur, which can present as an emergency.[2]

A systemic approach to the evaluation of dogs with suspected prostatic TCC is recommended to rule out other causes of prostatic disease such as benign prostatic hypertrophy, prostatitis, prostatic cysts or abscess and other neoplasias. Physical examination of the prostate gland is best achieved by a combination of rectal and abdominal palpation. The prostate on rectal palpation in dogs with prostatic TCC is typically large, firm, irregular, nodular, and/or asymmetric.[5] A non-atrophic prostate felt on rectal examination in a neutered dog should be considered abnormal and may be compatible with prostatic neoplasia or other prostatic disease.[5] Sublumbar lymphadenopathy may be detected on rectal palpation. Anemia, leukocytosis, hypercalcemia and elevated bone alkaline phosphatase activity may be apparent.[5] Pyuria, bacteriuria and dysplastic urinary epithelial cells may be identified by urinalysis or urine culture.[5] There is one report of a dog with prostatic carcinoma that had neoplastic cells in the peripheral circulation which is very rare.[5]

Imaging should include evaluation of the prostate as well as the regional lymph nodes and lungs for evidence of regional and distant metastasis. Evidence of an enlarged prostate may be visible on abdominal radiographs and there may be evidence of mineralization within the prostate.[2] Multifocal, irregularly shaped, parenchymal mineral densities are most commonly seen with prostatic carcinoma, but this change has also been identified in chronic prostatitis.[2] Evidence of periosteal reactions on the lumbar vertebrae (typically the fourth through seventh lumbar vertebrae), femur or pelvic bones, or sublumbar or retroperitoneal lymphadenopathy may be noted on abdominal radiographs.[5] Prostatic TCC that metastasizes to bone most commonly has an osteoproductive component but may also be osteolytic, osteoproductive or mixed.[5] Contrast studies such as retrograde urethrography may show irregularities in the prostatic urethra and contrast material that was refluxed may be found in the prostatic mass.[2] However, presence of contrast medium reflux is not specific for prostatic neoplasia.[2] Abdominal

ultrasonography can be useful to further evaluate the prostate, urethra, bladder, regional lymph nodes and cranial abdominal organs.

Obtaining tissue samples for histopathology analysis is considered the gold standard for diagnosis of canine prostatic TCC.[2] Cytological evaluation of material from the prostate can be useful in the differentiation of neoplasia of the prostate from other prostatic diseases. In one report, TCC of the prostatic urethra in two dogs appeared cytologically similar to prostatic adenocarcinoma.[2] Techniques that have been evaluated for the diagnosis of prostatic TCC include ejaculation, traumatic catheterization, prostatic massage, prostatic wash, ultrasound guided fine needle aspirate (FNA) cytology and prostatic biopsies.[2] Cytological evaluation of samples by FNA may prove challenging as it can be difficult to differentiate dysplastic epithelial cells from neoplasia therefore obtaining tissue sample biopsies for histopathology is preferred.[2] In one study, conflicting results between cytology and histopathology in prostatic neoplasia occurred in 20% of the cases.[2] Risks of obtaining a histological diagnosis include hemorrhage, trauma to the urethra and tumor seeding. Histological grading of canine prostate carcinoma is not commonly performed as there is no evidence it provides useful prognostic information.[2] A large study of 76 dogs with prostate carcinoma, including transitional cell carcinoma defined several histological sub-types of canine prostatic carcinomas, but did not find differences in survival time among the different morphological patterns.[3]

Without treatment, the prognosis for dogs with prostatic TCC is poor.[3] These tumors are highly metastatic and as noted previously, most dogs are diagnosed at advanced stages. Median survival times for dogs without therapy are often less than 30 days.[2] In one study of 76 dogs, a median survival time of approximately 21 days was reported, with most dogs euthanized at the time of diagnosis due to quality of life concerns.[2] If treatment is attempted, it is directed towards local disease control as well as control of regional and distant metastasis. Currently there is no standard of therapy for canine prostatic carcinoma, although treatment is largely considered palliative. The use of non-steroidal anti-inflammatory drugs (NSAIDs) is often recommended as palliative therapy.

Therapeutic options for managing local disease include prostatectomy, radiation therapy and medical management.[2,3] Surgery is generally considered to be a palliative procedure and the goals of surgery are to minimize clinical signs secondary to the primary tumor while maintaining normal urethral function.[2] Prostatectomy should be considered for dogs with diffuse intra-capsular tumors or an intra-capsular tumor surrounded by normal prostatic tissue with no evidence of metastasis.[3] Total prostatectomy has not been widely adopted for treating canine prostate carcinomas because it is associated with a high rate of postoperative morbidity, in particular urinary incontinence and because it is not clear that this surgical procedure will improve survival.[2] In a prospective randomized study of 21 dogs, 10 dogs had a total prostatectomy (TP) and 11 dogs had subtotal intracapsular prostatectomy (SIP).[3] Post-operative survival was longer for dogs in the SIP group than the TP group (mean 112 days vs. 20 days) and dogs in the SIP group had a decreased rate of postoperative complications.[3] Two of the 11 dogs in the SIP group and seven dogs in the TP group were euthanized within two weeks of surgery due to severe urinary complications.[3]

If the prostate tumor is causing urethral obstruction, palliative measures may be attempted to alleviate the obstruction. Placement of a cystostomy tube allows urinary diversion and bladder emptying, however due to the presence of the mass, stranguria and incontinence may persist.[2] While cystostomy tubes are well tolerated, careful patient and owner selection are necessary. Owners should be informed of possible complications of cystostomy tube placement including ascending urinary tract infections and tumor dissemination.[2] Palliative stenting of the urethra in the obstructed area is a reasonable alternative to cystostomy tubes. In one study, with eight dogs that had a urethral stent, the complication rate was low (only in two dogs), and the procedure immediately alleviated the obstruction in all dogs.[2] Median survival time in this study was short, at 20 days.[2] Treatment with effective adjunctive therapy such as cyclooxygenase (COX-2) inhibitors and earlier placement could make placement of cystostomy tubes or palliative urethral stents more beneficial to canine patients with prostatic TCC.[2]

Photodynamic therapy (PDT) is a localized treatment reported in one dog for treating prostatic carcinoma.[3] Following treatment, clinical signs of hematuria and a bloody preputial discharge resolved

and the prostate remained stable in size for at least six months.[3] A more recent study showed rapid local recurrence in a dog with prostatic carcinoma, despite intra-operative PDT after a partial prostatectomy, most likely due to insufficient light penetration.[3] Challenges in delivering a homogenous dose may limit the utility of PDT in advanced canine prostatic carcinomas. Currently, PDT remains investigational and not widely available.

Although optimal dose and fractionation are unknown, radiation therapy may be useful in the palliation of clinical signs related to local prostatic neoplasia as well as to palliate painful skeletal metastases.[2] In an early study of ten dogs treated with 20-30 Gy intraoperative orthovoltage radiation therapy, the prostate was radiated in nine dogs and affected regional lymph nodes were treated in three dogs.[2] The median survival time of the nine treated dogs with prostatic carcinoma was 114 days.[2] The results of radiation therapy have been disappointing and severe adverse effects have developed. It is clear that when radiating the urinary tract, prostate gland, and pelvic region, the total dose and dose per fraction must be carefully considered to avoid serious complications.[3] Complications of radiation therapy to the pelvic region have been described in a total of 66 dogs in two studies.[3] A high rate (30-50%) of complications were reported and included chronic colitis, gastrointestinal stricture or perforation, necrotic drainage/ulceration in the skin and subcutaneous tissues within the radiation field, urinary bladder thickening, chronic cystitis, urethral stricture, ileosacral osteosarcoma and perianal pain.[3] While many of these side effects did not affect survival, they did affect patient quality of life and owner expense.

The benefit of systemic therapy to manage canine prostatic carcinomas, especially TCC, is unclear. Recently, there has been interest in the anti-cancer effect of NSAIDs for a variety of prostatic carcinomas.[2] The precise mechanisms involved in tumor response to NSAIDs, especially carcinomas are not clearly defined, but are likely multi-factorial and may involve COX-2 inhibition and consequent inhibition of angiogenesis, stimulation of apoptosis, alterations in immune function and other mechanisms.[2] It was demonstrated in one study that there was a clear survival benefit in dogs with prostate carcinomas treated with a NSAID such as piroxicam or carprofen compared to those left untreated (6.9 months vs. 0.7 months).[2] Other palliative treatment options that may benefit canine patients with skeletal metastasis include bisphosphonates.[3] Bisphosphates are osteoclast inhibitors that are an integral part of the management of skeletal metastasis in men with prostate carcinomas and appear to have similar benefits in dogs with skeletal metastasis.[3]

Transitional cell carcinoma of the ductus deferens

Diseases of the ductus deferens (DD) in dogs have rarely been reported in the veterinary literature. Transitional cell carcinoma of the DD has been reported in one dog.[6] This dog was a neutered male with a one year history of recurring urinary tract infections.[6] Although no gross hematuria was reported, the dog had stranguria, which resolved upon treatment with NSAIDs and appropriate antimicrobials based on the multiple urine cultures and antibiotic sensitivity tests.[6] The stranguria would reoccur within days after discontinuation of treatment. On rectal examination, a firm, slightly prominent symmetric prostate was palpated with no signs of pain.[6] Abdominal ultrasonography revealed a normal sized prostate with irregular margins.[6] The prostate also had numerous mineralized foci.[7] A tubular structure that was 4 cm long and 1 cm in diameter was also identified between the bladder and the colon.[6] Although the origin of this structure was not clearly identified with abdominal ultrasonography, the distal part of the structure appeared to be connected to the prostate.[6] It was found on exploratory laparotomy that the right DD was dilated and fluid-filled and extended caudally into the prostate parenchyma.[6] Dilation of the DD of the canine patient is not commonly reported in the veterinary literature. In the one reported canine patient, the dilated DD appeared as a fluid-filled structure dorsal to the bladder.[8] If distention or dilation of the DD is observed, an underlying prostatic disease should be suspected.[7]

Histologically, the right DD from this dog had an irregular lumen, with irregular, disorganized transitional epithelium with extension into adjacent smooth muscle.[6] The DD is normally lined by pseudostratified columnar epithelium, whereas transitional epithelium lines the bladder and the prostatic urethra.[6] Therefore, TCC could not be a primary tumor of the DD. It is possible in this case, that local proliferation of prostatic TCC led to tumor invasion of the DD, considering that there is continuity

between the prostatic urethra and the epithelium of the ductus deferens.[6] It could also be speculated that TCC was seeded in the DD via urine.[6] At the time of straining, because of the increased pressure in the prostatic urethra, urine could have been pushed into the DD.[6]

Eight months after diagnosis of TCC in the right DD, the dog had urinary incontinence and developed severe hindlimb lameness.[6] Euthanasia was elected because the dog was no longer responding to palliative therapy with NSAIDs and antimicrobials, and because metastasis to bone was suspected.[6] Because normal DD is rarely seen during abdominal ultrasound in dogs, identification of a tubular, fluid-filled structure dorsal to the bladder may indicate an abnormal DD.[7] Transitional cell carcinoma of the DD should be included in the differential diagnosis of affected patients with clinical signs of the reproductive and urinary tracts.[6]

Vaginal and vulvar transitional cell carcinoma

Vaginal and vulvar tumors account for 2 to 3% of all canine tumors.[9] In a study of 2,917 tumor bearing dogs, 56 (2%) had tumors of the vagina and/or vulva.[10] These usually occur in middle aged to older intact female dogs.[5] The majority of the tumors at this site are benign.[5] In a survey of a total of 3,073 tumor bearing female dogs there were 85 (2.8%) dogs with tumors of the vagina or vulva with the majority (78%) of the tumors diagnosed as leiomyoma.[5] Leiomyomas are benign tumors of smooth muscle origin. In a review of 99 dogs with vulvar or vaginal tumors there were 72 benign and 27 malignant tumors.[9] The malignant tumors included TCC, leiomyosarcomas, mast cell tumors and squamous cell carcinoma. Transitional cell carcinomas arising from the bladder or urethra may manifest as a vaginal mass.[5] They can manifest near the urethral papilla and/or may develop on the labia of the vulva.[11] Presence of a mass protruding from the vulva is the most common clinical sign of vaginal and vulvar TCC, although vaginal bleeding or discharge is often noted.[11] Other clinical signs may include perineal swelling, stranguria, hematuria, excessive vulvar licking and dystocia.[11]

Evaluation of a suspected vaginal mass should include vaginal and rectal palpation and evaluation of the stage of the estrous cycle (vaginal cytology and serum progresterone level).[5] A presumptive diagnosis of vaginal and vulvar neoplasia may be made based on patient signalment and tumor location, although definitive diagnosis requires histopathology. Vaginoscopic examination and vaginal cytology are often the first steps performed in evaluation of vaginal and vulvar tumors.[11] Retrograde vaginography or urethrocystography may also be used to help delineate the extent of the mass.[11] For some tumor types including vaginal or vulvar TCC, cytology of the tumor may be diagnostic; alternatively, incisional biopsy may be performed. Additional diagnostics such as abdominal ultrasound and thoracic radiographs to assess for regional and pulmonary metastasis should be performed.[5]

Surgical excision is the treatment of choice for vaginal and vulvar TCC.[11] The majority of vaginal tumors, including TCC can be easily resected by episiotomy and local resection, but some more invasive tumors may require a more aggressive resection.[11] A combination of vulvovaginectomy and perineal urethrostomy allows resection of more extensive vaginal and/or vulvar tumors.[11] These surgical techniques allow resection of the distal urethra if necessary and dogs maintain urinary continence.[11] Chemotherapy is warranted in the management of metastatic vaginal and/or vulvar TCC, incompletely excised or inoperable tumors of the vagina or vulva.[5] The goal of chemotherapy would be to delay or prevent the onset of metastatic disease and try to temporarily decrease the size of the tumor. Radiation therapy can be used as a palliative treatment option and may help decrease discomfort associated with the mass. The prognosis for vaginal and/or vulvar TCC must be considered poor due to high rates of local recurrence and metastasis.

Conclusion

The actual incidence of TCC of the reproductive tract in dogs is rare. Most TCCs of the canine reproductive tract involve the prostate. Canine prostatic carcinomas including TCC, have served as an important model for studying human prostatic disease.[3] The canine prostate gland and the human prostate have many morphological and functional similarities.[3] The dog is also one of the few domestic species to develop spontaneous prostate neoplasia, resulting in the substantial interest of the dog as a model for

prostatic carcinoma in men.[3] Human prostatic neoplasia is the second most frequently diagnosed cancer and the sixth most common fatal cancer among men worldwide and its incidence is climbing.[2] This increase appears to be due to a combination of lifestyle and environmental factors as well as heightened public awareness. However, the incidence of prostate carcinoma is much lower in dogs.[3] Using the dog as a model for human prostate cancer presents the important challenge that most early stage prostate carcinomas in men are highly androgen dependent.[3] Unlike humans, dogs with prostate carcinoma usually present with advanced disease that does not respond to androgen deprivation therapy.[2] High grade prostate cancer in men behaves similarly to the disease in dogs, with significant local invasion and the tendency to metastasize.[3] Comparable to humans, affected dogs often develop osteoblastic bone metastasis in the pelvis and/or lumbar spine and present with associated pain or neurological deficits.[3] Other clinical signs such as weight loss, lethargy, abnormal urination and/or defection are also similar among humans and canines.

It is apparent that better methods of early detection and more effective therapies including palliative options are needed for prostatic cancer, including TCC in dogs and advanced prostate carcinoma in men.[3] Dogs with prostate neoplasia are relevant models for the disease in humans and pre-clinical studies of new diagnostics and therapies in dogs may benefit both humans and dogs with prostate neoplasia.[3] It has been shown that it would appear appropriate to limit exposure to lawn chemicals especially in breeds with high risk for TCC.[2] The owners of high risk breeds, such as the Shetland sheepdog and Scottish terrier should be informed of the TCC risk and be encouraged to take note of urinary tract signs if they should occur.[2]

References

1. Knapp DW, McMillan SH: Tumors of the urinary system. In: Withrow SJ, Vail DM, Page RL, editors. Small animal clinical oncology. 5th ed. St. Louis: Elsevier Saunders Co; 2013. p. 572-582.
2. Lawrence JA, Saba CF: Tumors of the male reproductive system. In: Withrow SJ, Vail DM, Page, RL, editors. Small animal clinical oncology. 5th ed. St. Louis: Elsevier Saunders Co; 2013. p 557-571.
3. LeRoy BE, Northrup N: Prostate cancer in dogs: comparative and clinical aspects. Vet J 2009;180:149-162.
4. Krawiec DR, Heflin D: Study of prostatic disease in dogs: 177 cases (1981–1986). J Am Vet Med Assoc 1992;200:1119-1122.
5. McEntee MC: Reproductive oncology. Clin Tech Small Anim Pract 2002;17:133-149.
6. Guerin VJ, Visser KW, L'Eplattenier HF, et al: Transitional cell carcinoma involving the ductus deferens in a dog. J Am Vet Med Assoc 2012;204: 446-449.
7. Bradburry CA, Westropp JL, Pollard RE: Relationship between prostatomegaly, prostatic mineralization, and cytologic diagnosis. Vet Rad Ultrasound 2009;50:167-171.
8. Kim B, Kawashima A, Ryu JA, et al: Imaging of the seminal vesicle and vas deferens. Radiographics 2009;29:1105-1121.
9. Brodey RS, Roszel JF: Neoplasms of the canine uterus, vagina, and vulva: a clinicopathologic survey of 90 cases. J Am Vet Med Assoc 1967;151:1294-1307.
10. Brodey RS: Canine and feline neoplasia. Adv Vet Sci Comp Med 1970;14: 309-354.
11. Saba CF, Lawrence JA: Tumors of the female reproduction system. In: Withrow SJ, Vail DM, Page RL, editors. Small animal clinical oncology. 5th ed. St. Louis: Elsevier Saunders Co; 2013. p. 532-537.

Comparison of canine thyroid hormone values between laboratories

Margaret V. Root Kustritz
College of Veterinary Medicine, University of Minnesota, St. Paul, MN

Abstract

Blood was drawn from 10 dogs, serum harvested and frozen, and shipped to two laboratories, both of which measured total and free thyroxine (T4 and fT4), total and free triiodothyronine (T3 and fT3), thyroglobulin autoantibody (TgAA), and thyroid stimulating hormone (TSH). Information was collected about the dogs including past thyroid hormone testing, thyroid supplementation and other medications, chronic disease conditions including allergic dermatitis, and diet. Values differed between laboratories in four of six dogs with complete data (67%). One laboratory recommended thyroxine supplementation for four of the dogs; the author of the study did not recommend thyroxine supplementation for any of the dogs based on thyroid hormone results and clinical presentation.

Keywords: Canine, thyroid, hypothyroidism

Introduction

Thyroxine (T4) is the hormone secreted by the thyroid gland. It is broken down by type I iodothyronine deiodinase to form bioactive triidothyronine (T3).[1] Hypothyroidism may be due to inadequate stimulation of pituitary thyroid hormone secretion by thyroid stimulating hormone (TSH) from the hypothalamus, insufficient secretion of T4, or inadequate conversion of T4 to T3. Hypothyroidism is the most common endocrinopathy of dogs, with reported prevalence of 0.2 to 0.8%.[2,3] Clinical manifestations include lethargy, weight gain, coat changes, intolerance for cold, unwillingness to exercise, variation in mentation, and neuropathies.[2,4,5] Diagnosis is complicated by variation in circulating thyroid hormones in the absence of thyroid disease. Activity of iodothyronine deiodinase may be decreased in animals with severe illness, fasting or cachexia, or severe liver disease.[6] Examples of other factors that may alter serum concentrations of T4 or T3 include breed, age, gender, reproductive status, concurrent use of drugs, and presence of non-thyroidal illness.[2,5,7-17]

Hypothyroid dogs have less circulating T4, both free and bound, and due to lack of feedback from those hormones, have more circulating TSH. Accurate testing requires assay in serum of free (unbound) T4 (fT4) and TSH. The assay for fT4 has a sensitivity of 0.89 to 0.98, a specificity of 0.93, and an accuracy of 0.95 for assessment of hypothyroidism in dogs.[18,19] The TSH assay is less sensitive, specific, and accurate, but if both assays are considered together, accuracy is 100%.[20]

Several large testing laboratories provide thyroid panels to give veterinarians and their clients a more definitive idea of the animal's thyroid hormone status. Most panels include assay for total T4, fT4, bound and free T3 (total T3 and fT3), and TSH. Some panels also include measurement of autoantibodies against thyroglobulin (TgAA) or against T3 and T4 (AAT3 and AAT4, respectively). Laboratories often provide interpretations with these assay results and differ greatly in the recommendations for treatment made.

The goal of this study was to compare values for concentration of total T4, fT4, total T3, fT3, TgAA, and TSH in serum between two different veterinary laboratories.

Materials and methods

Animals were enrolled from the population of dogs at the University of Minnesota College of Veterinary Medicine. Serum was harvested and frozen, and then shipped to two commercial laboratories for assessment. Information was collected regarding previous thyroid hormone testing, thyroid hormone supplementation or other medications received, chronic disease problems including allergic dermatitis, diet, and clinical signs of hypothyroidism including weight gain, lethargy, symmetrical alopecia, and heat-seeking behavior.

Results

Five spayed female and five castrated male dogs were enrolled in the study. Breeds represented were golden retriever (3), Labrador retriever, Siberian husky, Alaskan malamute, American Staffordshire terrier, English setter, Cavalier King Charles spaniel, and Olde English Bulldog. Age ranged from 1.9 to 10.4 years (6.3 ± 3.2 yrs [mean ± SD]).

Actual values from the two laboratories used are not reported. Concentrations were determined using different testing modalities and values were reported using different units and laboratory-specific reference ranges. Insufficient serum from four dogs was submitted, precluding analysis of those samples at one laboratory. Results were classified as normal or abnormal. Thyroid hormone results and specific information about each dog are listed in the Table.

Discussion

Dog breeders express concerns about evaluation of the thyroid status in their breeding bitches and stud dogs because of the many reports in the popular literature linking hypothyroidism to reproductive dysfunction in dogs.[21] There are few reports substantiating such concerns and pathology in reproductive tissues secondary to hypothyroidism may be less likely because the uterus and testes do not rely on thyroid hormone for oxygen consumption as do most other tissues.[3,22,23] Studies evaluating semen quality in male dogs with experimentally induced hypothyroidism failed to show any decline in libido or semen quality even in the presence of significant clinical manifestations of hypothyroidism.[24] Similarly, a long-term study in bitches with experimentally induced hypothyroidism demonstrated decreased puppy birth weight and prolonged parturition but no change in estrous cyclicity, pregnancy rate, litter size, or gestation length.[25,26] Despite this, and perhaps with valid concern that induced models of disease may not mimic spontaneous disease, many dog breeders ask their veterinarian to test their dogs for hypothyroidism in the absence of clinical manifestations of disease or treat their dog despite lack of evidence that the dog is hypothyroid.

Hypothyroidism may be misdiagnosed or mistreated in several ways. Veterinarians may make the mistake of assaying only T4, and using a low result as evidence for hypothyroidism.[27] Veterinarians and breeders may make the mistake of treating clinically normal dogs with "low normal" assay results, or treating empirically without any testing. A significant risk of hyperthyroidism exists if supplementation is provided unnecessarily; clinical manifestations of hyperthyroidism in dogs include agitation, tachypnea, tachycardia, atrial flutter, and syncope.[28]

None of the enrolled in this study was suspected of having hypothyroidism based on history or physical examination findings. Of the six dogs in this study with results from both laboratories, four (67%) had differing results for at least one parameter. Of the 10 dogs in the study, one laboratory recommended thyroid hormone supplement for four. Of these four dogs, three were recommended to be supplemented because of low thyroid hormone concentrations and normal TSH concentration. One of these dogs was on medications and had chronic disease that may have accounted for the change in thyroid hormone concentrations, although there are studies documenting that neither mild osteoarthritis nor use of non-steroidal anti-inflammatory drugs, including carprofen, are associated with changes in thyroid hormone concentration.[29,30]

The recommendation to supplement the fourth dog was based solely on presence of elevated TgAA concentrations. Some authors suggest that elevation in TgAA occurs early in disease, as an indication of presence of autoimmune destruction of the gland before there are other discernible changes in function.[31] One study demonstrated elevated concentrations in TgAA in several breeds predisposed to hypothyroidism but did not show subsequent progression to a clinical hypothyroid state, as tracked by owner questionnaires.[32] There is no evidence in the veterinary literature that supplementation in the presence of elevated TgAA only somehow alters trajectory of the disorder. In human medicine, among antibodies evaluated, TSH receptor blocking antibodies are considered likely to be associated with disease and TgAA is considered valuable only as diagnostic tool in childhood autoimmune thyroiditis.[33] The author of this study recommends thyroid hormone supplementation only if T4 is decreased and TSH is elevated in the absence of other causes of thyroid hormone concentration change and if clinical

manifestations of hypothyroidism are present, and therefore, did not recommend thyroid hormone supplementation for any of these dogs.

Conclusion

Thyroid hormone concentrations in a given dog may differ depending on the laboratory used, and interpretations from some laboratories may differ from recommendations in the veterinary literature. Veterinarians are encouraged to develop a relationship with one laboratory whose testing regimen they trust, and to work with colleagues at that laboratory in considering test results to make joint decisions about patient care based not only laboratory findings but also on history and physical examination findings and assessment of clinical evidence of disease.

References

1. Schoenmakers CHH, Pigmans IGAJ, Visser TJ: Species differences in liver type I iodothyronine deiodinase. Biochim Biophys Acta 1992;1121:160-166.
2. Panciera DL: Hypothyroidism in dogs: 66 cases (1987-1992). J Am Vet Med Assoc 1994;204:761-767.
3. Scott-Moncrief JC: Hypothyroidism: In: Ettinger SJ, Feldman EC, editors. Textbook of veterinary internal medicine. St Louis: Saunders; 2010. p. 1751-1761.
4. Vitali CL, Olby NJ: Neurologic dysfunction in hypothyroid, hyperlipidemic Labrador Retrievers. J Vet Intern Med 2007;21:1316-1322.
5. Nesbitt GH, Izzo J, Peterson L, et al: Canine hypothyroidism: A retrospective study of 108 cases. J Am Vet Med Assoc 1980;177:1117-1122.
6. Tepperman J: The thyroid. In: Metabolic and endocrine physiology. Chicago: Yearbook Medical Publishers;1980. p. 160.
7. Panciera DL, Refsal KR, Sennello KA, et al: Effects of deracoxib and aspirin on serum concentrations of thyroxine, 3,3,3'-triiodothyronine, free thyroxine, and thyroid-stimulating hormone in healthy dogs. Am J Vet Res 2006;67:599-603.
8. Gaughan KR, Bruyette DS: Thyroid function testing in Greyhounds. Am J Vet Res 2001;62:1130-1133.
9. Kantrowitz LB, Peterson ME, Melian C, et al: Serum total thyroxine, total triiodothyronine, free thyroxine, and thyrotropin concentrations in dogs with nonthyroidal disease. J Am Vet Med Assoc 2001;219:765-769.
10. Urhausen C, Seefeldt A, Eschricht FM, et al: Concentrations of prolactin, LH, testosterone, TSH and thyroxine in normospermic dogs of different breeds. Reprod Domest Anim 2009;44 (Suppl 2):279-282.
11. Gottschalk J, Einspanier A, Ungemach FR, et al: Influence of topical dexamethasone applications on insulin, glucose, thyroid hormone and cortisol levels in dogs. Res Vet Sci 2011;90:491-497.
12. Torres SMF, Feeney DA, Lekcharoensuk C, et al: Comparison of colloid, thyroid follicular epithelium, and thyroid hormone concentrations in healthy and severely sick dogs. J Am Vet Med Assoc 2003;222:1079-1085.
13. Hill RC, Fox LE, Lewis DD, et al: Effects of racing and training on serum thyroid hormone concentrations in racing Greyhounds. Am J Vet Res 2001;62:1969-1972.
14. Kantrowitz LB, Peterson ME, Trepanier LA, et al: Serum total thyroxine, total triiodothyronine, free thyroxine, and thyrotropin concentrations in epileptic dogs treated with anticonvulsants. J Am Vet Med Assoc 1999;214:1804-1808.
15. Nelson RW, Ihle SL, Feldman EC, et al: Serum free thyroxine concentration in healthy dogs, dos with hypothyroidism, and euthyroid dogs with concurrent illness. J Am Vet Med Assoc 1991;198:1401-1407.
16. Musser E, Graham WR: Familial occurrence of thyroiditis in purebred Beagles. Lab Anim Care 1968;18:58-68.
17. Conaway DH, Padgett GA, Bunton TE, et al: Clinical and histological features of primary progressive, familial thyroiditis in a colony of Borzoi dogs. Vet Pathol 1985;22:439-446.
18. Peterson ME, Melian C, Nichols R: Measurement of serum total thyroxine, triiodothyronine, free thyroxine, and thyrotropin concentrations for diagnosis of hypothyroidism in dogs. J Am Vet Med Assoc 1997;211:1396-1402.
19. Muth Beale K, Keisling K, Forster-Blouin S: Serum thyroid hormone concentrations and thyrotropin responsiveness in dogs with generalized dermatologic disease. J Am Vet Med Assoc 1992;201:1715-1717.
20. Scott-Moncrieff JC, Nelson RW, Bruner JM, et al: Comparison of serum concentrations of thyroid-stimulating hormone in healthy dogs, hypothyroid dogs, and euthyroid dogs with concurrent illness. J Am Vet Med Assoc 1998;212:387-391.
21. Dodds WJ, Laverdure DR: The canine thyroid epidemic. Wenatchee (WA): Dogwise Publishing; 2011.
22. Segalini V, Hericher T, Grellet A, et al: Thyroid function and infertility in the dog: a survey in five breeds. Reprod Domest Anim 2009;44(Suppl 2):211-213.
23. Peter AT, Gaines JD, Smith CL: Association of weak estrual signs and irregular estrous cycles with hypothyroidism in a bitch. Can Vet J 1989;30:957-958.
24. Johnson C, Nachreiner R, Mullaney T: Effect of experimentally induced thyroid insufficiency on reproduction function in male dogs. Proc Annu Meet Soc for Therio 1995; p. 236-240.

25. Panciera DL, Purswell BJ, Kolster KA, et al: Reproductive effects of prolonged experimentally induced hypothyroidism in bitches. J Vet Intern Med 2012;26:326-333.
26. Panciera DL, Purswell BJ, Kolster KA: Effect of short-term hypothyroidism on reproduction in the bitch. Theriogenology 2007;68:316-321.
27. Shiel RE, Sist MD, Nachreiner RF, et al: Assessment of criteria used by veterinary practitioners to diagnose hypothyroidism in sighthounds and investigation of serum thyroid hormone concentrations in healthy Salukis. J Am Vet Med Assoc 2010;236:302-308.
28. Fine DM, Tobias AH, Bonagura JD: Cardiovascular manifestations of iatrogenic hyperthyroidism in two dogs. J Vet Cardiol 2010;12:141-146.
29. Paradis M, Sauve F, Charest J, et al: Effects of moderate to severe osteoarthritis on canine thyroid function. Can Vet J 2003;44:407-412.
30. Sauve F, Paradis M, Refsal KR, et al: Effects of oral administration of meloxicam, carprofen, and a nutraceutical on thyroid function in dogs with osteoarthritis. Can Vet J 2003;44:474-479.
31. Graham PA, Refsal KR, Nachreiner RF: Etiopathologic findings of canine hypothyroidism: Vet Clin North Am Small Anim Pract 2007;37:617-631.
32. Ferm K, Bjornerfeldt S, Karlsson A, et al: Prevalence of diagnostic characteristics indicating canine autoimmune lymphocytic thyroiditis in giant schnauzer and hovawart dogs. J Sm Anim Pract 2009;50:176-179.
33. Brown RS: Autoimmune thyroiditis in childhood. J Clin Res Pediatr Endocrinol 2012. doi:10.4274/jcrpe.855. Accessed January 2, 2013.

Table: Thyroid hormone results and demographic data for 10 dogs (N = normal, L = low, H = high)

DOG	1	2	3	4	5	6	7	8	9	10
BREED	Olde English Bulldog	Alaskan malamute	Golden retriever	Golden retriever	Golden retriever	Cavalier King Charles spaniel	English setter	American Staffordshire terrier	Labrador retriever	Siberian husky
AGE (YRS)	1.9	2.3	2.7	5.0	5.8	6.9	8.8	9.2	9.7	10.4
GENDER	F/S	M/N	M/N	F/S	M/N	F/S	F/S	M/N	M/N	F/S
LAB 1 T4	N	***L***	L	L	N	N	N	N	N	N
LAB 2 T4	--	***N***	--	--	**N**	**N**	--	**N**	**N**	--
LAB 1 fT4	N	***L***	L	L	N	N	N	N	N	N
LAB 2 fT4	--	***N***	--	--	**N**	**N**	--	**N**	**N**	--
LAB 1 T3	N	***L***	L	L	***L***	N	L	***L***	***L***	L
LAB 2 T3	--	***N***	--	--	***N***	**N**	--	***N***	***N***	--
LAB 1 fT3	N	N	N	N	N	N	N	N	N	N
LAB 2 fT3	--	--	--	--	--	--	--	**N**	**N**	--
LAB 1 TgAA	N	N	N	N	***N***	H	N	N	N	N
LAB 2 TgAA	--	**N**	--	--	***H***	**H**	--	**N**	**N**	--
LAB 1 TSH	N	N	N	N	N	N	N	***H***	N	N
LAB 2 TSH	--	**N**	--	--	**N**	**N**	--	***N***	**N**	--
LAB 1 RECOMMEND-ATION	All levels normal, retest in 1 year	T4 low, T3 and TSH likely spurious. Begin supplementation with thyroxine.	T4 low, T3 and TSH likely spurious. Begin supplementation with thyroxine.	T4 low, T3 and TSH likely spurious. Begin supplementation with thyroxine.	T3 spurious, retest in 1 year	High TgAA indicative of autoimmune thyroiditis. Begin supplementation with thyroxine.	T3 spurious, retest in 1 year	T3 and TSH likely spurious, retest in 1 year	T3 spurious, retest in 1 year	T3 spurious, retest in 1 year
THYROID HORMONES MEASURED IN THE PAST?	No	No	No	No	Unknown	Yes, normal	Yes, normal	Yes, normal	Yes, normal	No
RECEIVING THYROID SUPPLEMENT?	No	No	No	No	No	No	No	No	No	No

OTHER MEDICATIONS?	Heartgard Plus™	No	Interceptor™, Frontline™, diphenhydra-mine	No	Heartwor m preventive and flea control	carprofen	Interceptor ™ prednisone	Interceptor ™	prednisone, fluoxetine	Heartgard™ , Frontline™
CHRONIC DISEASE?	No	No	Hip dysplasia (unmedicated)	No	No	No	Hip dysplasia	No	No	No
ALLERGIC DERMATITIS?	No	No	Yes	No	No	No	Yes	Yes (seasonal)	Yes	No
DIET?	Wellness Supermi x 5 Healthy Weight	Purina Pro Plan Weight Managemen t	Purina Large Breed	Purina ProPla n	Purina Senior	Purina Adult Performanc e	Purina Joint Mobility	Wellness Senior	Purina OM or Weight Managemen t	Purina JD
SIGNS OF HYPOTHYROIDISM ?	No	No	No	No	No	No	No	Slow weight loss, some heat-seeking	No	No

Retrospective evaluation of breeding management data and breeding type and correlation with successful breeding of bitches

Margaret V. Root Kustritz, Raeana Rice
College of Veterinary Medicine, University of Minnesota, St. Paul, MN

Abstract

Data were retrieved from medical records for breeding management of 576 dogs of 88 breeds. Pregnancy rate was highest with natural service and with surgical insemination with either chilled or frozen semen. Pregnancy rate was lower for vaginal insemination with any type of semen. Age of the bitch at the time of breeding was associated overall with success, with younger bitches (4.4 ± 1.7 years) more likely to be successfully bred than older bitches (5.2 ± 1.7 years) (p=0.04). For all semen types, litter size was significantly greater with surgical insemination than with vaginal insemination (p=0.0004). Serum progesterone concentration on the day of the luteinizing hormone (LH) surge was 3.1 ± 1.0 ng/ml, with a range of 1.3 to 6.2 ng/ml (n = 63). In general, a lower progesterone concentration was associated with success when using fresh semen and a higher progesterone concentration was associated with success when using frozen semen. This was a directed study project for a senior veterinary student. Collecting these data and talking through how the cases had been managed and significance of the findings were excellent learning opportunities for this student.

Keywords: Breeding management, progesterone, LH, litter size, artificial insemination

Introduction

Several factors unique to bitches make breeding management more challenging than in other species. Because we have limited access to the ovaries physically or visually, we must infer what is happening on the ovaries by diagnostic testing. Bitches ovulate an immature oocyte, which must undergo one more meiotic division before it can be fertilized. For this reason, optimal breeding is offset from ovulation day. Many of the diagnostic tests used approximate the day of ovulation but only endocrine assays provide the general practitioner with any accuracy in prospectively determining ovulation day.

Luteinizing hormone is the stimulus for ovulation in bitches. It is secreted pulsatilely, with a large, single peak. Ovulation occurs 36 to 50 hours later.[1] Direct measurement of LH is the most definitive diagnostic test available. Unfortunately, commercial LH assays are not readily available and turn-around time makes their use impractical for clinical cases. In-house LH assays are intermittently available. In-house LH assays do not provide quantitative measurement of serum LH concentration but only differentiate low from high (less than or greater than 1 ng/ml [3.1 nmol/L]).

Progesterone is the hormone assay most commonly used for assessment of ovulation date in bitches. Because bitches undergo preovulatory luteinization, rise in serum progesterone concentrations can be used to infer date of the LH peak and prospectively predict ovulation day. In general, progesterone concentration on the day of the LH peak will be about 2.0 ng/ml and on ovulation day from 4 to 10 ng/ml.[2] Some veterinarians prefer not to look at individual values but instead watch for a sudden increase in progesterone concentration by 3 ng/ml or more from one day to the next, denoting that as ovulation day. Some denote the first day progesterone concentration is 5 ng/ml or greater as ovulation day.[3] Finally, some veterinarians look for an absolute value of progesterone at the time of breeding, with anecdotal reports of breeding optimized when progesterone is greater than 10 ng/ml or when progesterone is 15 ng/ml. The authors are unaware of scientific studies documenting clinical significance of the latter method.

Type of semen (fresh, chilled, or frozen) and insemination technique (natural service, vaginal insemination, or transcervical or surgical intrauterine insemination) impact pregnancy rate and litter size in bitches, as does age of the bitch. Breeding management with hormone assay is required to optimize timing of breeding, especially as more sophisticated breeding techniques are used. Commonly recommended timing of insemination for a single breeding is two days post-ovulation for fresh or chilled

semen and three to four days post-ovulation for frozen semen.[4] Fertilization has been reported to occur 3.5 to 7.5 days after the LH peak in bitches.[5]

Vaginal insemination most commonly is done using an infusion pipette. More specialized equipment has been described but has not been demonstrated to increase pregnancy rate or litter size.[6,7] Transcervical insemination can be done as a blind technique, using a rigid catheter, or may be done with assistance of a long, narrow endoscope used to see the cervix and aid passage of a polypropylene urinary catheter through the cervix.[8] There is one report of laparoscopic intrauterine insemination in bitches.[9] Surgical insemination is done via laparotomy with exteriorization of the uterus and direct injection of semen into the uterine body.

Besides timing of breeding, number of times bred has a significant effect on pregnancy rate, with a greater number of breedings associated with increased pregnancy rate and litter size.[10-13] For the purposes of this review, the authors will not attempt to pull out details of breeding timing from the information presented, as it is variably reported. Similarly, pregnancy rate will be the term used in this discussion. Some papers report conception rate, although that terminology is inappropriate considering inability of determining actual number of conceptuses in bitches. Some papers report whelping rate and in this review, those are described as pregnancy rate, recognizing some inaccuracy due to possible pregnancy loss when considering litter size.

This study is an evaluation of success in providing breeding services for one veterinary clinic in the United States, using data drawn from the medical records.

Materials and methods

Banked serum samples were donated by a local practitioner who does extensive reproductive work. Progesterone had been assayed using chemiluminescence, either at a commercial laboratory or in-house. Biweekly quality control sample measurements were performed using both internal and external controls. Complete medical records were maintained for all bitches. For about one-fourth of these bitches, samples were collected daily from some point in proestrus through insemination.

Data were retrieved from all records for each breeding management episode including breed; age; dates of progesterone sampling and breeding; LH assays performed, if any; type of breeding performed, number of breedings/cycle; semen quality; success of breeding; and litter size.

Statistical analysis was performed using descriptive statistics, analysis of variance (ANOVA), Student's t-testing for non-categorical data, and calculation of the Pearson's correlation coefficient. Significance was set at $p<0.05$.

Results

Data were available for 576 dogs of 88 breeds. The Labrador retriever was best represented (27.1%). The next most common breeds, in order, were the German shepherd dog, Australian shepherd, golden retriever, border collie, Bernese mountain dog, Rottweiler, bichon frise, German wirehaired pointer, American Staffordshire terrier, Cavalier King Charles spaniel, and English setter. Mean age of bitches bred was 4.5 ± 1.8 years (n=311).

Overall pregnancy rate varied with type of breeding (Table 1). Pregnancy rate was highest with natural service and with surgical insemination with either chilled or frozen semen. Pregnancy rate was lower for vaginal insemination with any type of semen. Pregnancy rate for transcervical insemination with chilled semen was low, as was sample size.

Age of the bitch at the time of breeding was associated overall with success, with younger bitches (4.4 ± 1.7 years) more likely to be successfully bred than older bitches (5.2 ± 1.7 years) ($p=0.04$). There were no specific breeding types that were more or less successful relative to age of the bitch ($p=0.72$).

Litter size varied with age of the bitch and with type of breeding. Age of the bitch was not associated with litter size overall but for the most common breed, the Labrador retriever, there was a positive association of age with decreasing litter size ($r=0.19$), with the effect enhanced specifically for surgical insemination with frozen semen ($r=0.34$). Association with type of breeding and litter size was

significant (p=0.02; Table 2). For all semen types, litter size was significantly greater with surgical insemination than with vaginal insemination (p=0.0004).

Serum progesterone concentration on the day of the LH surge was 3.1 ± 1.0 ng/ml, with a range of 1.3 to 6.2 ng/ml (n = 63). Concentration of progesterone on the first breeding day was not different between successful (13.7 ± 8.5, n=192) and unsuccessful attempts (12.5 ± 7.4, n=56) for all types of breeding together (p=0.35). Concentration of progesterone on the first breeding day was different between successful and unsuccessful attempts for some types of breeding (Table 3). In general, a lower progesterone concentration was associated with success when using fresh semen and a higher progesterone concentration was associated with success when using frozen semen.

Discussion

This was a directed study project for a senior veterinary student. Collecting these data and talking through how the cases had been managed and significance of the findings were excellent learning opportunities for this student. Specific competencies demonstrated by the student included ability to describe use of measurement of LH or progesterone in serum for breeding management, pros and cons of various types of semen and insemination techniques, and how to evaluate a given bitch to optimize pregnancy rate and litter size. She also was able to provide the clinic with the information that their success rate with surgical insemination of chilled and frozen semen approximated natural service, suggesting they are doing a good job providing reproductive services. A fair number of bitches were lost to follow-up and the student is considering recommendations for record keeping at the clinic to better capture these data.

Age of the bitch is associated with pregnancy rate. In one study, bitches over six years of age had lower whelping rates than did younger bitches.[11] This trend of greater likelihood of success when breeding bitches at a younger age was upheld in our study.

Reported pregnancy rate for natural service in dogs ranges from 84.5% to 100%.[9,14-17] Fresh semen usually is introduced vaginally; reported pregnancy rates are 47.8 to 100%.[15,18] There was one report of pregnancy rate of 25% with vaginal insemination of fresh semen but this low value was attributed to significant backflow of semen along the catheter used, with subsequent loss through the vulva.[16] Pregnancy rate for transcervical insemination with fresh semen is 65.2% to 90%.[15,16,19] There are reports of surgical insemination with fresh semen. In those studies, pregnancy rate was 83.7 to 100%.[20,21] Because spermatozoa that are used immediately after collection are viable for a prolonged period, and because the goal when determining insemination technique for a given type of semen is to use the safest and least invasive method with the lowest risk of side-effects that is still likely to achieve the desired results, surgical insemination would only be used if there was proven subfertility with vaginal insemination.[20,22]

For chilled semen, pregnancy rate with vaginal insemination is 33 to 89% and with transcervical insemination is 65.6%.[15,23]

Frozen semen is viable for only a short time after thawing, so intrauterine insemination is recommended. Pregnancy rate for vaginal insemination with frozen semen is generally low, 10 to 60%, with one report of 80% success rate in a group of five dogs.[10,11,15,18,24,25] Pregnancy rate may be increased by inseminating multiple times and by introducing prostatic fluid vaginally after the semen is deposited.[26] Reported pregnancy rate for transcervical insemination with frozen semen is 52.0 to 100%.[10-12,15,16,24,25,27] Reported pregnancy rate for surgical insemination with frozen semen is 70.8 to 89.4%.[21,28]

Pregnancy rates in this group of bitches agreed with what has been demonstrated previously in the literature. The small number of transcervical inseminations with chilled semen precluded meaningful interpretation of that data. In general, there is less information about success rate with chilled semen in the veterinary literature.

Litter size is associated with size of the breed, with larger breed bitches having larger litters.[21,29-31] Correlation between litter size and weight of the dam is 0.83.[32] Another breed-specific component that may impact litter size is inbreeding. Inbreeding coefficient is negatively correlated with litter size in bitches, suggesting that choosing for specific desirable characteristics may unintentionally be associated

with choosing for decreased reproductive success.[33,34] Pedigree analysis to determine inbreeding coefficient was not performed in this study.

Age of bitch has been associated with litter size in bitches. In one study in which age was not associated with litter size, bitches were not bred beyond about four years of age.[14] In a study evaluating litters registered with the American Kennel Club, 10% of bitches showed a decline in litter size after their second litter or three years of age.[31] In another study, litter size decreased after the fifth parity or about six years of age.[35] As might be expected, age-related decline in litter size occurred at a younger age in larger breed bitches.[31] Data from the best-represented breed in this study, the Labrador retriever, agree with these trends from the literature.

Litter size is impacted by timing of breeding, by type of breeding, and by number of breedings. Litter size is optimized by breeding two to three days post-ovulation.[36] In one study evaluating longevity of canine ova post-ovulation, it was determined that litter size declined significantly by eight days post-ovulation.[37] Cervical closure occurs at about six days post-ovulation but even introduction of semen directly into the uterus does not promote normal litter size, suggesting that aging of the ova or asynchrony with the intrauterine environment play a role.[37,38]

In one study evaluating birth of 10,810 litters from 224 bitches, litter size was greater for those bitches bred by natural service than with any form of artificial insemination (AI).[29] In other studies, litter size was demonstrated to be greater with fresh semen than with frozen semen.[13,21,31] In that study, litter size decreased by 0.4 pups with fresh semen AI and by 1.3 pups with frozen semen AI.[29] Average litter size with vaginal insemination with fresh semen is 5.8 and with transcervical insemination is 6.5.[15] There is one report of transcervical insemination with fresh semen, with litter size of 5.0 pups.[19] Average litter size with vaginal insemination of chilled semen is 5.8 and with transcervical insemination is 6.4.[15] Average litter size with vaginal insemination of frozen semen is 4.7 and with transcervical insemination is 5.0 to 6.9 pups.[15,28] Finally, it has been demonstrated that litter size is positively associated with number of inseminations.[13] In this study, we demonstrated greater litter size with surgical insemination than with vaginal insemination, and greater litter size with surgical insemination than with natural service. This was an uncontrolled population, with many dogs that bred by natural service in this community not available for analysis, suggesting that these data are skewed by the population seen.

Assays available for measurement of progesterone in serum or plasma include radioimmunoassay (RIA), chemiluminescence (CLA) assay, and enzyme-linked immunosorbent assay (ELISA). Enzyme-linked immunosorbent assay has been demonstrated to be about 90% accurate, compared to RIA.[39] Comparison of RIA to CLA demonstrated correlation of 0.98, with some studies suggesting consistently higher values with RIA than with CLA measurement.[40-42] There is value in handling the sample consistently and in using the same kind of assay throughout a given breeding management episode. Use of evacuated serum separator tubes has been associated with higher concentrations measured by CLA, some studies suggest that letting the serum sample sit on the clot may be associated with artificially low serum progesterone as the hormone molecule adheres to red blood cells in the clot, some studies suggest generally higher values using RIA compared to CLA, and finally, some studies have demonstrated diurnal variation in progesterone secretion in bitches.[42] Sample handling was consistent within the facility and CLA was used for assay of all samples in this study.

Serum progesterone concentration is reported to vary from 2 to 4 ng/ml or 1 to 1.9 ng/ml on the day of the LH peak.[2,43] Data from this study agree with data from the first study cited. Ovulation occurs two days after the LH peak, with reported values of 38.0 ± 4.4 hrs with ultrasound observation and a range of 24 to 72 hours with laparoscopic observation.[44-46] Serum progesterone concentration on ovulation day has been reported to vary from 3 to 10 ng/ml.[2,43,47] Because spermatozoa that have been manipulated are less likely to be viable long-term than are spermatozoa that have not been chilled or frozen, it is not surprising that success in this study was associated with relatively lower progesterone concentrations for fresh semen, presumably associated with breeding a bit earlier and relying on long-term viability of spermatozoa, and with relatively higher concentrations for frozen-thawed semen.

Conclusion

Retrospective evaluation of data permitted this veterinary clinic to assess its success rate relative to the veterinary literature and to fine-tune breeding management and record keeping. Data such as these provide insights to guide client decision making.

Acknowledgment

Source of funding: The Theriogenology Foundation

References

1. Concannon PW, McCann JP, Temple M: Biology and endocrinology of ovulation, pregnancy and parturition in the dog. J Reprod Fertil 1989;Suppl 39:3-25.
2. Johnston SD, Root MV: Serum progesterone timing of ovulation in the bitch. Proc Annu Meet Soc Therio 1995; p. 195-203.
3. Concannon PW: Reproductive cycles of the domestic bitch. Anim Reprod Sci 2011;124:200-210.
4. Tsumagari S, Ichikawa Y, Toriumi H, et al: Optimal timing for canine artificial insemination with frozen semen and parentage testing by microsatellite markers in superfecundency. J Vet Med Sci 2003;65:1003-1005.
5. Badinand F, Fontbonne A, Maurel MC, et al: Fertilization time in the bitch in relation to plasma concentration of oestradiol, progesterone and luteinizing hormone and vaginal smears. J Reprod Fert 1993;47:63-67.
6. Nizanski W: Intravaginal insemination of bitches with fresh and frozen-thawed semen with addition of prostatic fluid: Use of an infusion pipette and the Osiris catheter. Theriogenology 2006;66:470-483.
7. Kojima Y, Kawakami S, Shino M, et al: A small trial on a new insemination technique with fresh dog semen using the artificial penis. J Reprod Dev 1996;42:j39-j41.
8. Wilson MS: Endoscopic transcervical insemination in the bitch. In: Concannon PW, England G, Verstegen J, et al, editors. Recent advances in small animal reproduction. Ithaca (NY): www.ivis.org, A1232.1203.
9. Silva LDM, Onclin K, Snaps F, et al: Laparoscopic intrauterine insemination in the bitch. Theriogenology 1995;43:615-623.
10. Thomassen R, Farstad W, Krogenaes A, et al: Artificial insemination with frozen semen in dogs: A retrospective study. J Reprod Fertil 2001;Suppl 57:341-346.
11. Thomassen R, Sanson G, Krogenaes F, et al: Artificial insemination with frozen semen in dogs: a retrospective study of 10 years using a non-surgical approach. Theriogenology 2006;66:1645-1650.
12. Farstad W, Andersen Berg K: Factors influencing the success rate of artificial insemination with frozen semen in the dog. J Reprod Fertil 1989;Suppl 39:289-292.
13. Linde-Forsberg C, Forsberg M: Results of 527 controlled artificial inseminations in dogs. J Reprod Fertil 1993;47:313-323.
14. England GCW, Russo M, Freeman SL: Follicular dynamics, ovulation and conception rates in bitches. Reprod Domest Anim 2009;44:53-58.
15. Linde-Forsberg C: Intra-uterine insemination in the dog using the Scandinavian transcervical catheter and comparison with other methods. In: Concannon PW, England G, Verstegen J, et al, editors. Recent advances in small animal reproduction. Ithaca (NY):www.ivis.org, A1207.0201.
16. Farstad W: Bitch fertility after natural mating and after artificial insemination with fresh or frozen semen. J Sm Anim Prac 1984;25:561-565.
17. Van Haaften B, Dieleman SJ, Okkens AC, et al: Timing the mating of dogs on the basis of blood progesterone concentration. Vet Rec 1989;125:524-526.
18. Silva LDM, Onclin K, Lejeune B, et al: Comparisons of intravaginal and intrauterine insemination of bitches with fresh or frozen semen. Vet Rec 1996;138:154-157.
19. Macedo SP, Malm C, Henry MRJM, et al: Endoscopic transcervical intrauterine artificial insemination in Labrador retriever bitches. Res Vet Sci 2012;92:494-500.
20. Brittain D, Concannon PW, Flanders JA, et al: Use of surgical intrauterine insemination to manage infertility in a colony of research German shepherd dogs. Lab Anim Sci 1995;45:404-407.
21. Burgess DM, Mitchell KE, Thomas PGA: Coeliotomy-assisted intrauterine insemination in dogs: A study of 238 inseminations. Aust Vet J 2012;90:283-290.
22. Goodman M: Just because I can, doesn't mean I should: the application of evidence-based medicine to small animal theriogenology. Clin Therio 2012;4:521-525.
23. Nishiyama T, Kinugasa T, Kimura T, et al: Determination of optimal time for mating by artificial insemination with chilled semen using luteinizing hormone surge as an indicator in Beagles. J Am Anim Hosp Assoc 1999;35:348-352.
24. Linde-Forsberg C, Strom Holst B, Govette G: Comparison of fertility data from vaginal versus intrauterine insemination of frozen-thawed dog semen: a retrospective study. Theriogenology 1999;52:11-23.
25. Rota A, Iguer-Ouada M, Verstegen J, et al: Fertility after vaginal or uterine deposition of dog semen frozen in a Tris extender with or without Equex STM paste. Theriogenology 1999;51:1045-1058.

26. Nothling JO, Gerstenberg C, Volkmann DH: Success with intravaginal insemination of frozen-thawed dog semen – a retrospective study. J S Afr Vet Assoc 1995;66:49-55.
27. Hayashi K, Morita R, Aso T, et al: Evaluation of transcervical insemination using frozen semen by flexible endoscope in dogs. J Vet Med Sci 2012 (in press).
28. Pretzer SD, Lillich RK, Althouse GC: Single, transcervical insemination using frozen-thawed semen in the Greyhound: a case series study. Theriogenology 2006;65:1029-1036.
29. Borge KS, Tonnessen R, Nodtvedt A, et al: Litter size at birth in purebred dogs – a retrospective study of 224 breeds. Theriogenology 2011;75:911-919.
30. Lyngset A, Lyngset O: Litter size in the dog. Nord Vet Med 1970;22:186-191.
31. Kelley RL: Canine reproduction management: Factors influencing litter size. Proc Annu Meet Soc for Therio; 2002.
32. Robinson R: Relationship between litter size and weight of dam in the dog. Vet Rec 1973;92:221-223.
33. Gresky C, Hamann H, Distl O: Influence of inbreeding on litter size and the proportion of stillborn puppies in Dachshunds. Berl Munch Tierarztl Wochenschr 2005;118:134-139.
34. Root Kustritz MV: Decreased litter size in the bitch. Proc Minn Vet Med Assoc 2007.
35. Mutembi HM, Mutiga ER, Tsuma VT: An epidemiological survey demonstrating decline in reproductive efficiency with age and non-seasonality of reproductive parameters in German shepherd bitches in Kenya. J S Afr Vet Assoc 2002;73:36-37.
36. Shimatsu Y, Yuzawa H, Aruga K, et al: Effect of time for mating and gestation length on reproductive efficiency in dogs. Reprod Domest Anim 2007;42:664-665.
37. Tsutsui T, Takahashi F, Hori T, et al: Prolonged duration of fertility of dog ova. Reprod Domest Anim 2009;44:230-233.
38. Verstegen JP, Silva LD, Onclin K: Determination of the role of cervical closure in fertility regulation after mating or artificial insemination in Beagle bitches. J Reprod Fertil 2001;Suppl 57:31-34.
39. Moxon R, Copley D, England GCW: Technical and financial evaluation of assays for progesterone in canine practice in the UK. Vet Rec 2010;167:528-531.
40. Kohen F, Kim JB, Lindner HR, et al: Development of a solid-phase chemiluminescence immunoassay for plasma progesterone. Steroids 1981;38:73-88.
41. DeBoever J, Kohen F, Vandekerckhoue D, et al: Solid-phase chemiluminescence immunoassay for progesterone in unextracted serum. Clin Chem 1984;30:1637-1641.
42. Hegstad-Davies RL: A review of sample handling considerations for reproductive and thyroid hormone measurement in serum or plasma. Theriogenology 2006;66:592-598.
43. Hewitt D, England G: Assessment of optimal mating time in the bitch. In Prac 2000;22:24-33.
44. Phemister RD, Holst PA, Spano JS, et al: Time of ovulation in the Beagle bitch. Biol Reprod 1973;8:74-82.
45. Hase M, Hori T, Kawakami E, et al: Plasma LH and progesterone levels before and after ovulation and observation of ovarian follicles by ultrasonographic diagnosis system in dogs. J Vet Med Sci 2000;62:243-248.
46. Wildt DE, Chakraborty PK, Panko WB, et al: Relationship of reproductive behavior, serum luteinizing hormone and time of ovulation in the bitch. Biol Reprod 1978;18:561-570.
47. Wright PJ: Practical aspects of the estimation of the time of ovulation and of insemination in the bitch. Aust Vet J 1990;68:10-13.

Table 1: Pregnancy rate by breeding type

TYPE	PREGNANCY RATE (%)	SAMPLE SIZE
NATURAL	85.2	27
FRESH VAG	61.3	31
CHILLED VAG	66.7	12
CHILLED TCI	33.3	3
CHILLED SX	81.8	11
FROZEN SX	84.9	86

Table 2: Litter size by breeding type

TYPE	OVERALL N	OVERAL LITTER SIZE
NATURAL	21	6.0 (2.3)
FRESH VAG	19	4.9 (2.1)
CHILLED VAG	8	4.4 (1.6)
CHILLED TCI	2	5.0
CHILLED SX	6	7.0 (3.9)
FROZEN SX	56	6.8 (2.6)

Table 3: Progesterone concentration in serum at time of first breeding by breeding type

TYPE	OVERALL N	OVERALL P4	SUCC N	SUCC P4	UNSUCC N	UNSUCC P4	P VALUE
NATURAL	41	7.0 ± 4.8	23	6.5 ± 4.7	4	6.2 ± 1.1	0.9
FRESH VAG	61	10.8 ± 6.7	19	8.1 ± 4.3	12	13.9 ± 8.9	0.02
CHILLED VAG	27	10.3 ± 5.6	8	9.6 ± 6.4	4	10.8 ± 5.7	0.76
CHILLED TCI	6	18.4 ± 10.9	1	12.6	2	12.0	NA
CHILLED SX	14	16.5 ± 5.3	9	18.3 ± 4.4	2	13.5	NA
FROZEN SX	120	21.6 ± 4.6	73	21.6 ± 4.2	13	19.1 ± 4.1	0.05

Succ = successfully bred, Unsucc = not successfully bred

Fungal diseases of the canine reproductive tract

Margaret V. Root Kustritz
College of Veterinary Medicine, University of Minnesota, St. Paul, MN

Abstract

Fungal diseases of the canine reproductive tract are uncommon. The most commonly described is blastomycosis, which is associated with disease in both male and female dogs. Secondary yeast infections of the reproductive tract, as are described in humans, are uncommon in dogs.

Keywords: Fungi, yeast, prostatitis, blastomycosis

Fungi are free-living organisms. They are heterotrophs, incapable of producing their own nutrients, which differentiates them from plants. Fungi release enzymes and absorb nutrients from their environment. Their cell wall is made of chitin, not of cellulose as is seen in plants, which makes them relatively resistant to microbial degradation and may make them more resistant to drug therapies that require uptake across the cell. Most are multicellular and filamentous. A group of fungal filaments are hyphae and groups of hyphae form a mycelium. Fungi may produce as much as one kilometer of hyphae in a single day in the right growing conditions.

Fungi may or may not exist as a single-celled yeast as part of their life cycle and may or may not form spores. There are two main phyla of fungi that are relevant to this discussion. These are the Ascomycota and the Basidiomycota. The Ascomycota include molds, yeasts, morels, truffles, and Dutch elm disease. This class of fungi is filamentous when growing and reproduces by producing spores called conidia at the end of modified hyphae called conidiophores. Yeasts are unicellular forms that reproduce by budding. Pathogenic fungi in this phylum include *Aspergillus* sp., *Blastomyces dermatitidis*, *Candida* sp., and *Sporothrix schenckii*. The basidiomycota include mushrooms and toadstools, rusts, smuts, stinkhorns, and shelf fungi. Most organisms in this phylum produce specialized club-shaped spores called basidia but the species that are pathogenic in small animal veterinary medicine do not. These species are *Cryptococcus* sp. and *Rhodotorula* sp., both of which exist primarily as yeasts.

There are relatively few reports in the literature of fungal diseases of the canine reproductive tract. Please note that this discussion will not include fungal diseases of dogs that have not been reported in the reproductive tract. These include histoplasmosis and coccidioidomycosis (Valley Fever).[1-3] Diseases are presented in alphabetical order, not in order of prevalence or clinical significance.

Aspergillosis

Aspergillus sp. are widespread in a variety of climates worldwide, with hundreds of species in the genus. It was named by an Italian priest who thought the shape of the spore-forming structure in this genus resembled the container used to sprinkle holy water, called an aspergillum.

Nasal aspergillosis is the most common type of disease reported in dogs and it usually is due to *Aspergillus fumigatus*. Disseminated fungal disease due to *Aspergillus* sp. is most commonly reported in German Shepherd dogs.[4,5] This breed predisposition may be due to abnormalities in amount or function of IgA with subsequent effects on mucosal immunity.[6-8] Cases most commonly are reported in California and in Australia.[4] Clinical presentation varies with organ system affected and may include gastrointestinal signs; pain, lameness and paraplegia; central nervous system signs; anorexia and muscle wasting; cutaneous edema, and ocular signs. Pyometra due to aspergillosis has been reported as has disseminated aspergillosis including uterine infection apparently brought on by the stress of breeding.[4,5] There is a report of puppies either stillborn or that died shortly after birth due to diffuse aspergillosis. They were delivered by cesarean section to a bitch that developed neurologic signs due to diffuse aspergillosis and was euthanized shortly thereafter, leading the authors to deduce that transplacental transmission of the organism had occurred.[9] There is one report of orchitis and epididymal atrophy secondary to aspergillosis in a dog.[10]

Definitive diagnosis usually is made by identification of organisms on cytology or in urine sediment. Culture may be used to confirm the diagnosis. Serologic testing rarely is performed because of a high rate of false positive results in dogs with other systemic mycoses.[4,11] Changes on routine bloodwork often are unremarkable but may include a mature neutrophilia on complete blood count (CBC) and increases in blood urea nitrogen, alkaline phosphatase, and alanine aminotransferase.[4]

Amphotericin B is the treatment of choice, with itraconazole a secondary therapy if needed.[4] Prognosis for cure in cases of disseminated disease is guarded. There is one report of treatment of disseminated aspergillosis with fluconazole in which the dog showed initial improvement but eventually demonstrated progression of disease and was euthanized.[5] The male dog with orchitis was treated with itraconazole until culture of semen was negative but he failed to impregnate bitches after therapy and those bitches that he bred developed uterine disease of unreported etiology.[10]

Blastomycosis

Systemic infection with blastomycosis was first reported in humans in the late 1800's.[12] Blastomycosis is the most commonly reported infection of the canine reproductive tract. Overall incidence of blastomycosis in dogs is 0.2 to 1.4% per year in endemic areas.[13,14] *Blastomyces dermatitidis* is the causative organism. It exists in soil as a hyphal form and is most commonly found in the Mississippi, Missouri and Ohio river valleys and in the area of the Great Lakes. Other areas include the mid-Atlantic states and southeastern states, and southern Canadian waterways. In general, risk is increased in animals and humans with proximity to water.[15,16] Some reports suggest greater prevalence in some seasons of the year while others refute those findings.[14,15,17] There are reports of dogs infected with blastomycosis that had no history of having lived or traveled in endemic areas.[18] The organism grows best in moist, acidic soil containing decaying vegetation and animal feces. Spores form directly on the hyphae, not on a distinct fruiting body, and usually are transmitted to the animal by inhalation. This is facilitated by disruption of the soil.

Once spores are inhaled, the higher body temperature of the infected animal promotes development of the yeast form of the organism, with infection most commonly localizing in the respiratory tract. Direct penetration of the organism, usually associated with trauma, may permit development of localized disease peripherally.[1] With any route of exposure, there may be localized spread or systemic disease may be induced by spread through lymphatics or the bloodstream.[1]

Clinical disease is most common in young dogs (2-4 years of age) and in large breeds, especially in hounds and sporting breeds.[14,15] Reports differ regarding predisposition by gender, with some reporting no such predisposition and others reporting increased incidence in males.[1,13-15] Signs may not appear for weeks to months after exposure and include anorexia, depression, weight loss, fever, lymphadenopathy, and respiratory signs including dyspnea, tachypnea, and cyanosis. Other systems that may be involved include the eye, skin, and bone.[15] Central nervous system involvement is rare.[1]

Blastomycosis has been reported as a cause of orchitis and prostatitis in dogs.[13,15,19-22] Dogs presented for scrotal swelling, with or without palpable changes in the testes.[20] Of four dogs in one study with prostatic infection, three were asymptomatic.[19]

Mammary gland infection also has been reported.[15,23] Two of three dogs with mycotic mastitis in one case series also had lymphadenopathy and pulmonary disease.[23]

Definitive diagnosis is by histopathologic demonstration of the yeast, which is thick-walled, 8-12 micrometers in diameter, lacks a capsule, and may bud with daughter cells having a broad-based attachment to the primary yeast cell.[1,15] Direct tissue sampling (fine needle aspirate or biopsy) is superior to indirect samples (transtracheal wash, for example). The organism often is accompanied by granulomatous or pyogranulomatous inflammation.[21-24] Changes on routine bloodwork often are unremarkable; hypoalbuminemia is reported in about 75% of cases, hyperglobulinemia in about 50% of cases, and hypercalcemia in about 10% of cases. Normocytic, normochromic anemia and moderate leukocytosis may be noted on CBC. Organisms may be visible in urine sediment from dogs with prostate infection.[13,20] Characteristic radiographic findings in dogs with respiratory signs are a diffuse or nodular interstitial pattern or alveolar infiltration.[15] Characteristic radiographic findings in dogs with bone

involvement are osteolysis and periosteal proliferation.[1] Culture of the organism is not routinely done because the organism is slow-growing and culture puts laboratory personnel at risk. Serologic testing has been described but is not definitive.[25]

Treatment is curative in 70 to 75% of cases. Itraconazole is the treatment of choice for systemic disease when compared to ketoconazole and amphotericin B, although one author reported equal success with itraconazole alone or a combination of ketoconazole and amphotericin B and another study showed that while duration of therapy with itraconazole was shorter, longer term, equally successful therapy with fluconazole was less expensive.[15,26] Affected dogs should be treated for at least two months and until clinical signs have been resolved for one month. Those cases with the poorest prognosis are those with involvement of three or more body systems and those with severe respiratory infection. Mortality usually occurs within the first seven days of treatment secondary to the inflammatory response that accompanies simultaneous death of numerous organisms.[1]

A genetically engineered attenuated vaccine against *Blastomyces dermatitidis* has been described for use in dogs. This vaccine is not commercially available.[27]

Candidiasis

Candida sp. exist as yeasts and are common in the environment and on alimentary, upper respiratory, and genital mucosal surfaces of animals and humans, where they mostly exist as harmless commensals whose overgrowth is inhibited by presence of normal bacterial flora.[28] *Candida* sp. were identified in 6.4% of 100 vaginal samples and 2.2% of 93 preputial samples collected from healthy dogs.[29] *Candida albicans* causes candidiasis, or thrush, in people, generally manifested as inflammation of a mucosal surface such as the oral cavity or vagina. Predisposing factors for candidiasis in humans include being immunocompromised from disease or drug therapy, disruption to the mucosal surface, and prolonged antibiotic use.[30] Similar risk factors described in dog with urinary tract infection due to candidiasis include recent treatment with antibiotics or glucocorticoids, and concurrent disease, including diabetes mellitus.[31]

There are reports of localized candidiasis in non-healing ulcers of the genital mucosa.[28] The ulcer is covered by a white to grey plaque and has hyperemic margins.[28] There also are reports of urinary tract infections due to candidiasis and systemic candidiasis; some of those dogs had concurrent prostate disease.[32,33]

Candida sp. are visible as small, thin-walled, ovoid yeasts. Culture of the organism requires lysis centrifugation of the sample to cause release of the fungus from leukocytes. Because it is part of the normal flora, culture results must be interpreted carefully.

Lesions are treated topically with nystatin, gentian violet, or miconazole or amphotericin B antifungal creams or lotions.[28] Treatment for prostatic infection is not described.

Cryptococcosis

Cryptococcus sp. also are yeast forms; technically, any growth as hyphae of this organism are classified in a different genus. The yeast cell of this species is covered by a gelatin-like capsule, which contains factors that appear to increase virulence of the organism.

The most common clinical manifestation of cryptococcosis is multisystemic disease with central nervous system signs.[34] There are reports of urinary tract infections due to *Cryptococcus neoformans*, suggesting a possibility for infection of the reproductive tract in dogs.[32] There is one report of a puppy dying of disseminated cryptococcosis at two weeks of age. The organism could be cultured from the vulvar secretions of that pup's dam for a prolonged period but eventually cultures were negative and she went on to whelp a normal litter.[35]

Rhodotorulosis

Rhodotorula sp. are pigmented yeasts that ordinarily live in soil but can be found in moist home environments, especially in bathrooms. These are commensal organisms on moist skin. In humans, infection occurs in immunosuppressed individuals.[28] There is one report of urinary tract infection in a

dog and there is one report of epididymitis in a dog, both due to infection with *Rhodotorula mucilaginosa*.[32,36] When grown on Sabouraud's dextrose agar, the colonies have a distinct creamy orange to salmon color.

Sporotrichosis

Sporothrix schenckii is an important component of soil worldwide, acting as a decomposer of plant material. It is a hyphal form at environmental temperatures and a yeast form at body temperature. Because of its role in decomposition, it is readily found in landscaping materials including sphagnum moss, landscape timbers, and baled hay. It is most commonly transmitted as a pathogen through puncture wounds, with direct inoculation. Cutaneous and cutaneolymphatic disease are the most common manifestations with rare systemic disease in dogs.[37] The organism has been isolated from the testes of systemically affected dogs.[38] It is a polymorphic yeast, taking on round, oval, and cigar shapes, and is a difficult organism to identify on cytology as it requires special staining. Accuracy may be enhanced by concurrent immunohistochemistry and staining.[39] Diagnosis often requires culture of a deep tissue biopsy. Disease is treated with appropriate antibiotics for secondary skin infection and with a long-term course of supersaturated potassium iodide. Some dogs may show signs of iodine toxicity with therapy, including ocular and nasal discharge, dry coat with excessive scaling, vomiting, depression, and collapse. If these signs occur, ketoconazole or itraconazole may be used for therapy.[37]

References

1. Kerl ME: Update on canine and feline fungal diseases. Vet Clin North Am Small Anim Pract 2003;33:721-747.
2. Wolf AM: Histoplasmosis. In: Greene CE, editor. Infectious diseases of the dog and cat. Philadelphia: Saunders; 1988. p. 378-383.
3. Greene RT: Coccidioidomycosis. In: Greene CE, editor. Infectious diseases of the dog and cat, Philadelphia: Saunders; 1988. p. 391-398.
4. Day MJ: Canine disseminated aspergillosis. In: Greene CE, editor. Infectious diseases of the dog and cat, Philadelphia: Saunders; 1988. p. 409-413.
5. Walker JT, Frazho JK, Randell SC: A novel case of canine disseminated aspergillosis following mating. Can Vet J 2012;53:190-192.
6. Day MJ, Penhale WJ: An immunohistochemical study of canine disseminated aspergillosis. Aust Vet J 1991;68:383-386.
7. Littler RM, Batt RM, Lloyd DH: Total and relative deficiency of gut mucosal IgA in German shepherd dogs demonstrated by faecal analysis. Vet Rec 2006;158:334-341.
8. Day MJ: Low IgA concentration in the tears of German shepherd dogs. Aust Vet J 1996;74:433-434.
9. Elad D, Lahav D, Blum S: Transuterine transmission of Aspergillus terreus in a case of disseminated canine aspergillosis. Med Mycol 2008;46:175-178.
10. Siemieniuch MJ, Skarzynski DJ, Kozdrowski R: Aspergillosis of a dog genital tract – case report. Anim Reprod Sci 2009;112:164-171.
11. Garcia RS, Wheat LJ, Cook AK, et al: Sensitivity and specificity of a blood and urine galactomannan antigen assay for diagnosis of systemic aspergillosis in dogs. J Vet Intern Med 2012;26:911-919.
12. Gilchrist T: A case of blastomycetic dermatitis in man. John Hopkins Hosp Rep 1896;1:269.
13. Legendre AM: Blastomycosis. In: Greene CE, editor. Infectious diseases of the dog and cat, Philadelphia: Saunders; 1988. p. 371-377.
14. Rudmann DG, Coolman BR, Perez CM, et al: Evaluation of risk factors for blastomycosis in dogs: 857 cases (1980-1990). J Am Vet Med Assoc 1992;201:1754-1759.
15. Arceneaux KA, Taboada J, Hosgood G: Blastomycosis in dogs: 115 cases (1980-1995). J Am Vet Med Assoc 1998;213:658-664.
16. Reed KD, Meece JK, Archer JR, et al: Ecologic niche modeling of Blastomyces dermatitidis in Wisconsin. PLoS One 2008;3:1-7.
17. Baumgardner DJ, Paretsky DP, Baseman ZJ, et al: Effects of season and weather on blastomycosis in dogs: Northern Wisconsin, USA. Med Mycol 2011;49:49-55.
18. Pratt CL, Sellon RK, Spencer ES, et al: Systemic mycosis in three dogs from nonendemic regions. J Am Anim Hosp Assoc 2012;48:411-416.
19. Totten AK, Ridgway MD, Sauberli DS: Blastomyces dermatitidis prostatic and testicular infection in eight dogs (1992-2005). J Am Anim Hosp Assoc 2011;47:413-418.
20. Wilson RW, Van Dreumel AA, Henry JNR: Urogenital and ocular lesions in canine blastomycosis. Vet Pathol 1973;10:1-11.
21. Shull RM, Hayden DW, Johnston GR: Urogenital blastomycosis in a dog. J Am Vet Med Assoc 1977;171:730-735.

22. Reed LT, Balog KA, Boes KM, et al: Pathology in practice: Blastomycosis in a dog. J Am Vet Med Assoc 2010;236:411-413.
23. Ditmyer H, Craig L: Mycotic mastitis in three dogs due to Blastomyces dermatitidis. J Am Anim Hosp Assoc 2011;47:356-358.
24. Garma-Avina A: Cytologic findings in 43 cases of blastomycosis diagnosed ante-mortem in naturally-infected dogs. Mycopathol 1995;131:87-91.
25. Spector D, Legendre AM, Wheat J, et al: Antigen and antibody testing for the diagnosis of blastomycosis in dogs. J Vet Intern Med 2008;22:839-843.
26. Mazera ASW, Trepanier LA, Foy DS: Retrospective comparison of the efficacy of fluconazole or itraconazole for the treatment of systemic blastomycosis in dogs. J Vet Intern Med 2011;25:440-445.
27. Wuthrich M, Krajaejun T, Shearn-Bochsler V, et al: Safety, tolerability, and immunogenicity of a recombinant, genetically engineered, live-attenuated vaccine against canine blastomycosis. Clin Vacc Immunol 2011;18:783-789.
28 Greene CE, Chandler FW: Candidiasis, torulopsosis, and rhodotorulosis. In: Greene CE, editor. Infectious diseases of the dog and cat, Philadelphia: Saunders; 1988. p. 414-417.
29. Brito EHS, Fontenelle ROS, Brilhante RSN, et al: The anatomical distribution and antimicrobial susceptibility of yeast species isolated from healthy dogs. Vet J 2009;182:320-326.
30. Eckert LO, Hawes SE, Stevens CE, et al: Vulvovaginal candidiasis: clinical manifestations, risk factors, management algorithm. Obstet Gynecol 1998;92:757-765.
31. Pressler BM, Vaden SL, Lane IF, et al: Candida spp. urinary tract infections in 13 dogs and seven cats: Predisposing factors, treatment, and outcome. J Am Anim Hosp Assoc 2003;39:263-270.
32. Jin Y, Lin D: Fungal urinary tract infections in the dog and cat: a retrospective study (2001-2004). J Am Anim Hosp Assoc 2005;41:373-381.
33. Kuwamura M, Ide M, Yamate J, et al: Systemic candidiasis in a dog, developing spondylitis. J Vet Med Sci 2006;68:1117-1119.
34. Jacobs GJ, Medleau L: Cryptococcosis. In: Greene CE, editor. Infectious diseases of the dog and cat, Philadelphia: Saunders; 1988. p. 383-390.
35. Hodgin EC, Corstvet RE, Blakewood BW: Cryptococcosis in a pup. J Am Vet Med Assoc 1987;191:697-698.
36. Kadota K, Uchida K, Nagatomo T, et al: Granulomatous epididymitis related to Rhodotorula glutinis infection in a dog. Vet Pathol 1995;32:716-718.
37. Rosser EJ, Dunstan RW: Sporotrichosis. In: Greene CE, editor. Infectious diseases of the dog and cat, Philadelphia: Saunders; 1988. p. 399-402.
38. Madrid IM, Mattei AS, Fernandes CG, et al: Epidemiological findings and laboratory evaluation of sporotrichosis: a description of 103 cases in cats and dogs in southern Brazil. Mycopathol 2012;173:265-273.
39. Mirando LHM, Quintella LP, Menezes RC, et al: Evaluation of immunohistochemistry for the diagnosis of sporotrichosis in dogs. Vet J 2011;190:408-411.

Managing bovine trichomoniasis in the female

Misty A. Edmondson
Department of Clinical Sciences, College of Veterinary Medicine, Auburn University Auburn, AL

Abstract

Bovine trichomoniasis is a sexually transmitted disease caused by the extracellular protozoa *Tritrichomonas foetus*, an obligate parasite of the reproductive tract of cattle. Infections in cows cause endometritis, cervicitis, vaginitis which may result in early embryonic death, abortion, pyometra, fetal maceration, or infertility. The major economic losses associated with *T. foetus* are due to: 1) reduced calf crop due to early embryonic loss or abortion, 2) reduced weaning weight due to delayed conception, and 3) culling and replacement of infected cattle. Due to the inability to use efficacious drugs, such as the nitromidazoles, for control and prevention of *T. foetus* infections in food animals, most control efforts have targeted identification and elimination of positive bulls, systemic immunization of cows and bulls, and management strategies to prevent introduction of the organism into the herd. This paper will review trichomoniasis in the female and discuss pathogenesis of disease, transmission, consequences of infection, immunity, diagnostic techniques, and control and prevention strategies.

Keywords: *Tritrichomonas foetus*, trichomoniasis, bovine, cow, prevention, control

Introduction

Bovine trichomoniasis is a sexually transmitted disease caused by the extracellular protozoa *Tritrichomonas foetus*, an obligate parasite of the reproductive tract of the cow and the folds on the mucosal surfaces of the bull's penis and prepuce. Infected bulls are often asymptomatic carriers of *T. foetus*. However, these infected bulls are capable of transmitting the organism to a cow during coitus.[1] Infections in cows cause endometritis, cervicitis, vaginitis which may result in early embryonic death, abortion, pyometra, fetal maceration, or infertility.[1] The major economic losses associated with *T. foetus* are due to: 1) reduced calf crop due to early embryonic loss or abortion, 2) reduced weaning weight due to delayed conception, and 3) culling and replacement of infected cattle. Due to the inability to use efficacious drugs, such as the nitromidazoles, for control and prevention of *T. foetus* infections in food animals, most control efforts have targeted identification and elimination of positive bulls, systemic immunization of cows and bulls, and management strategies to prevent introduction of the organism into the herd.

Pathogenesis in the female

Life cycle

The life cycle of *T. foetus* is thought involve two forms 1) a tear-shaped trophozoite form and 2) a pseudocyst form. The trophozoite is 10-25μm long and possesses three posterior flagella, one anterior flagellum and an undulating membrane. Trophozoites multiply asexually through binary fission.[2] Pseudocysts usually appear as a result of unfavorable conditions; although, a small percentage of pseudocysts exist under normal conditions.[3] Pseudocysts occur when *T. foetus* trophozoites round up and internalize their flagella in response to assorted stimuli.[3-5] The pseudocyst form lacks a protective cyst wall and does not represent a true cyst form.[4]

Trophozoites of *T. foetus* are transmitted between cows and bulls during coitus and remain in the genito-urinary tract where they multiply by longitudinal binary fission. Under stressful conditions trophozoites will internalize their flagella and replication of the nuclei and other cellular structures will occur, resulting in a multinucleated pseudocyst form. When conditions become desirable once more, mononucleate trophozoites will bud from the pseudocyst. In bulls, infections are usually chronic and asymptomatic and often persist for the life of the animal. Infected cows will initially experience vaginitis which may or may not resolve spontaneously. In some cases, endometritis can occur resulting in complete sterility. Tritrichomonas infections may also result in fetal loss during pregnancy.[6]

Studies have revealed that pseudocyst formation and reversal can be rapidly and simply effected by certain cooling and warming patterns.[4] However, the induction of pseudocysts by chemicals, dependent of exposure time and concentration, can lead to an irreversible process that leads to the death of the cells.[7] Historically, there has been some uncertainty about whether pseudocysts represent a normal or infective form rather than a degenerative form. More recent research indicates that *T. foetus* is easily stimulated into the pseudocyst form and that these immotile pseudocysts are able to proceed with the process of adhesion to the vaginal epithelial cells.[5] In addition, it has been demonstrated that the pseudocysts are more cytotoxic when in contact with host cells when compared to trophozoites.[8]

Transmission

Cows become infected with *T. foetus* primarily through coital exposure with an infected bull. Subsequently, a mild vaginitis occurs that may go undetected. The organism gains entry into the uterine lumen via the cervix during estrus. Colonization of the entire reproductive tract with *T. foetus* occurs within one to two weeks.[9] Although, contaminated semen or contaminated insemination equipment may also be minor sources of infection.[1] Penetration of the vagina is seemingly necessary because swabbing the vulvar area with high numbers of organisms does not result in vaginal or uterine infection.[10] Infected cows conceive but infection causes endometritis, cervicitis, or vaginitis which results in death of the conceptus within the first half of gestation, abortion, pyometra, fetal maceration, or infertility.[1] These infected cows usually remain infertile for a period of two to six months. In heifers, the duration of infection is reported to be as short as 95 days[11] or as long as 22 months.[12] *Tritrichomonas foetus* has been detected in the reproductive tract for 13 to 28 weeks after experimental infection in heifers.[13]

Consequences of infection

T. foetus organisms arrive in the female reproductive tract concurrently with spermatozoa. However in most cases, fertilization occurs in spite of the presence of the pathogen. In vitro studies have demonstrated that fertilization and early embryonic development to the hatching stage (eight to ten days) are not significantly affected by simultaneous culture with *T. foetus*.[14] Conceptus deaths most commonly occur between 50-70 days of gestation. Therefore, the majority of pregnancy loss is during the fetal period (>42 days of gestation). Although unusual, occasional abortions can occur of fetuses greater than four months of gestation.

Most producers do not recognize a problem in the early breeding season as conception occurs normally. The conceptus in most infected cows typically survives long enough to release sufficient interferon tau to prevent the prostaglandin $F_{2\alpha}$-mediated lysis of the corpus luteum. Fetal death in infected cows occurs between seven to ten weeks of gestation. Death of the conceptus during the early stages of pregnancy results in a prolonged interestrous interval.[9,15] Due to abortions and subsequent immunity, the distribution of pregnancies is unusually skewed with a higher proportion of pregnancies conceived towards the end of the breeding season. Although in many progressively managed herds with a limited breeding season, the bulls may no longer be available by the time the cow aborts and clears the infection. Therefore, *T. foetus* infection in a herd may go unnoticed until the time of pregnancy diagnosis when a high percentage of females are diagnosed not pregnant. Pyometra, along with abortions, may be the first physical signs of *T. foetus* infection in a herd, but are thought to occur in less than five percent of infected cows.[16] Pyometra results as the corpus luteum of pregnancy is maintained with a large purulent response which may cause damage to the uterine endometrium.[17]

Most infected cows will clear the organism and develop short-lived immunity of six months to one year. However, carrier cows do occur and are capable of spreading the protozoa. In the case of carrier cows, a very small percentage of cows (<1%) in infected herds have been shown to remain infected throughout pregnancy and into the following breeding season. Thus, the carrier cow has the potential to be quite devastating to control efforts and emphasizes that control programs must focus on the entire herd, not just the bull.[9]

Pathologic changes have been reported in several late-term, *T. foetus* aborted fetuses.[18] The placentas had focal or diffuse invasion of the chorionic stroma by *T. foetus* as seen on hematoxylin and

eosin stained sections of placentas. There was also evidence of a moderate inflammatory cell infiltrate comprised mostly of mononuclear cells. Six of 11 fetuses that were examined had bronchopneumonia with identifiable trichomonads in the airways. Another examination of late term abortions associated with *T. foetus* found necrotizing enteritis and pyogranulamatous bronchopneumonia with tissue invasion by trichomonads. The exact mechanism that leads to the death of the conceptus is not fully understood although cytotoxic and hemolytic effects by *T. foetus* on mammalian cells have been described.[19]

Immunity

In the female, *T. foetus* induces inflammation of the mucosa of the vagina, the cervix, the endometrium and the oviductal mucosa. In the first one to two weeks after infection, neutrophils and eosinophils predominate; however, this is followed by a moderate to severe mononuclear infiltration of lymphocytes and plasma cells. Subepithelial and periglandular lymphoid nodules resembling lymphoid follicles begin to develop at almost six weeks after infection. In addition, there is also an apparent degranulation of mast cells between six to nine weeks after infection.[20]

T. foetus specific IgA and IgG_1 antibodies are detectable in uterine and vaginal secretions by the fifth to sixth week after infection. The IgA antibodies do not kill the organisms but may be responsible for immobilization and agglutination of parasites as well as preventing adhesion of the organisms to the mucosal surfaces. The IgG_1 antibodies are presumed to facilitate complement mediated lysis of the parasites as well as opsonization and enhanced phagocytic killing by neutrophils or macrophages. Immunity following natural infection and clearance of *T. foetus* is short-lived with females becoming susceptible within a year, in time for the following breeding season. Because *T. foetus* is an extracellular pathogen, the immune response from the host is predominately humoral and the result of the short-lived immunity. The uterine mucosal inflammation that is seen with infection may allow systemically derived IgG and complement to gain access to the lumen of the uterus and, thus, clear the organism. A relative lack of IgG from the vagina or possibly blocking of IgG effects by vaginal IgA binding of organisms may help explain the carrier state that can be seen in infected herds.[20]

Diagnosis

The comparison of diagnostic assays for detection of *T. foetus* infections has primarily focused on the bull. Isolation of *T. foetus* from the female is reported to be less sensitive when compared with techniques used for bulls.[21,22] In one study, the InPouch™ TF system (BioMed Diagnostics, Inc; White City, OR) was more effective than Diamond's medium (88% versus 68%) in detecting heifers that had been experimentally infected with *T. foetus*.[23] The accuracy of prevalence in the cow most likely depends on the timing of sampling relative to exposure. The immune response in females begins to eliminate the infection within eight to ten weeks after exposure in unvaccinated females.[13] Therefore, cultures from females are best performed before the infection is possibly eliminated by the immune response.[23]

Sample handling is also crucial for accurate detection of *T. foetus*. When evaluating temperature and media type it has been found that when laboratory or field isolates were cultured in Diamond's medium or InPouch™ TF, all cultures were positive for *T. foetus* when maintained for up to four days at either 22° or 37°C. However, samples maintained at 4°C or less resulted in inconsistent sensitivity.[24] It is important to remember that time, temperature, type of isolate, and type of medium all have an effect on the sensitivity of *T. foetus* culture.

Microscopic evaluation of cultured organisms is not sufficient to differentiate *T. foetus* from nonpathogenic intestinal or coprophilic trichomonads (*Pentatrichomonas hominis*, *Tetratrichomonas* spp., etc).[25] Therefore, several conventional and real-time polymerase chain reaction (PCR) assays have been developed for the definitive diagnosis of trichomoniasis, and this methodology has demonstrated some advantages over culture.[25] However, accurate PCR results are directly related to the quality of the sample, which can be affected by transport condition parameters such as temperature and time of transport to the laboratory. There have been a number of issues that have limited the sensitivity of various conventional PCR assays for the detection of *T. foetus*. These problems include DNA degradation, accumulation of inhibitory compounds, sample contamination, and unexpected amplification products.[26] One study

demonstrated a decrease in sensitivity of PCR testing with samples that were stored for five days or more. However, PCR was in 100% agreement with culture as long as the PCR was performed within 24 hours of the sample being submitted.[26]

A more recent study evaluated the effect of different simulated transport conditions on samples containing *T. foetus* for the diagnosis of trichomoniasis using culture and quantitative PCR (qPCR).[25] This study demonstrated that transport temperatures of 4-20°C for 1-3 days before culture reduced or temporarily inhibited parasite replication but maintained viability. Samples tested by either culture or qPCR would have been expected to give positive results. However, diagnosis of trichomonads by both methods was negatively affected when specimens were maintained at transport temperatures of 42°C for 24 hours or more. This study emphasizes the importance of ensuring that clinical samples arrive at the diagnostic laboratory within 24-48 hours and of avoiding temperature transport conditions above 37°C in order to achieve an accurate diagnosis of *T. foetus*. The effects of high incubation temperatures on culture and real-time PCR for *T.* foetus have also been evaluated following inoculation into the InPouch™ TF system.[27] This study showed that *T. foetus* was detectable microscopically in inoculated pouches incubated at 37°C regardless of exposure time (one, three, six and 24 hours), whereas those samples incubated at 46.1°C detected *T. foetus* only after one and three hours of incubation. *T. foetus* was detected in samples incubated at 54.4°C after only one hour. Testing using real-time PCR for all inoculated media samples (37°C, 46.1°C, and 54.4°C at one, three, six and 24 hours) produced positive results for all inoculated media samples. This study suggests that samples collected for culture alone should be protected from high temperatures.

Prevention and control

One complicating factor with bovine trichomoniasis in the United States is the lack of effective treatments with U.S. Food and Drug Administration approval. Historically, the most successful treatment for bulls with trichomoniasis was systemic treatment with nitromidazole derivatives.[28] Currently, the use of nitromidazole derivatives is illegal in food-producing animals in the U.S., and no effective alternative treatments are available. The lack of effective, approved therapies for bovine trichomoniasis emphasizes the need for appropriate preventive and control measures. Prevention of trichomoniasis includes the following recommendations: 1) avoid movement of animals (co-grazing, leasing of bulls, good fences); 2) utilize artificial insemination if possible; 3) use a defined breeding season and cull all non-pregnant females after the breeding season; 4) purchase virgin bulls and heifers as replacements; 5) test all bulls for *T. foetus* prior to introduction into the herd and maintain a young population of bulls; and 6) breed purchased cows and heifers in a separate herd.[9]

Once *T. foetus* has been confirmed in a herd, there are additional measures that should be considered in order to "clean up" the herd. These measures include 1) testing and culling all infected bulls and purchasing *T. foetus* negative bulls; 2) intense management of bulls so that smaller breeding units are used and bulls are bred to the same cattle until trichomoniasis is under control; 3) create high and low risk herds; and 4) vaccinate all herd females with an approved *T. foetus* vaccine.[9] Vaccination is an important aspect of any control program as it has been shown to reduce pregnancy wastage associated with *T. foetus* infection in cattle herds. Currently, TrichGuard® (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) is the only commercially available vaccine licensed by the USDA for the control of trichomoniasis in the United States. TrichGuard® is a proprietary vaccine that is a Freund's adjuvant killed *T.* foetus-derived vaccine that requires two doses subcutaneous injections administered two to four weeks apart with the last injection to be given four weeks prior to the breeding season.[9] One study compared pregnancy and calving rates between beef heifers vaccinated with TrichGuard® and control heifers after heifers were exposed to *T. foetus* infected bulls and intravaginally inoculated with a large number (10 million) of *T. foetus* organisms.[29] Twice as many vaccinated heifers calved when compared to control heifers (61% versus 31%). Thus, the vaccine appeared to offer at least some protection against *T. foetus*.

Conclusion

Trichomoniasis can be an economically devastating infection in cattle herd with losses due to reduced calf crop due to early embryonic loss or abortion, reduced weaning weight due to delayed conception, and culling and replacement of infected cattle. Carrier females and concerns with diagnostic sampling and testing have made the control of trichomoniasis in cattle even more complex. Control and prevention of *T. foetus* infections in cattle must focus on identification and elimination of positive cows and bulls, systemic immunization of cows and bulls, and management strategies to prevent introduction of the organism into the herd.

References

1. BonDurant RH: Pathogenesis, diagnosis and management of trichomoniasis in cattle. Vet Clin North Am Food Anim Pract 1997;13:345-361.
2. Levine ND: Veterinary protozoology. Ames(IA): Iowa State University Press; 1985.
3. Pereira-Neves A, Ribeiro KC, Benchimol M: Pseudocysts in trichomonads - new insights. Protist 2003;154:313-329.
4. Granger BL, Warwood SJ, Benchimol M, et al: Transient invagination of flagella by *Tritrichomonas foetus*. Parasitol Res 2000;86:699-709.
5. Mariante RM, Lopes LC, Benchimol M: *Tritrichomonas foetus* pseudocysts adhere to vaginal epithelial cells in a contact-dependent manner. Paristol Res 2004;92:303-312.
6. Barratt JLN, Harkness J, Marriott D, et al: The ambiguous life of *Dientamoeba fragilis*; the need to investigate current hypotheses on transmission. Parasitology 2011;138:557-572.
7. Mariante RM, Guimaraes CA, Linden R, et al: Hydrogen peroxide induces caspase activation and programmed cell death in the amitochondrial *Tritrichomonas foetus*. Histochem Cell Biol 2003;120:129-141.
8. Pereira-Neves A, Nascimento LF, Benchimol M: Cytotoxic effects exerted by *Tritrichomonas foetus* pseudocysts. Protist 2012;163:529-543.
9. Rae DO, Crews JE: *Tritrichomonas foetus*. Vet Clin North Am Food Anim Pract 2006;22:595-611.
10. Clark BL, Dufty JH, Parsonson IM: Studies on the transmission of *Tritrichomonas foetus*. Aust Vet J 1977;53:170-172.
11. Parsonson IM, Clark BL, Dufty JH: Early pathogenesis and pathology of *Tritrichomonas foetus* infection in virgin heifers. J Comp Pathol 1976;86:59-66.
12. Alexander GI: An outbreak of bovine trichomoniasis in Queensland and its control. Aust Vet J 1953;29:61-69.
13. Skirrow SZ, BonDurant RH: Induced *Tritrichomonas foetus* infection in beef heifers. J Am Vet Med Assoc 1990;196:885-889.
14. Bielanski A, Ghazi DF, Phipps-Todd B: Observations on the fertilization and development of preimplantation bovine embryos in vitro in the presence of *Tritrichomonas foetus*. Theriogenology 2004;61:821-829.
15. BonDurant RH: Diagnosis, treatment, and control of bovine trichomoniasis. Compend Cont Educ Pract Vet 1985;7:S179-S188.
16. Kimsey PB: Bovine trichomoniasis. In: Morrow DA, editor. Current therapy in theriogenology. 2nd ed. Philadelphia: WB Saunders Co; 1986. p.275-279.
17. BonDurant RH, Honinberg BM: Trichomonads of veterinary importance. In: Kreier JP, editor. Parasitic protozoa, volume 9. 2nd ed. San Diego: Academic Press, Inc.; 1994. p. 136-162.
18. Rhyan JC, Stackhouse LL, Quinn WJ: Fetal and placental lesions in bovine abortion due to *Tritrichomonas foetus*. Vet Pathol 1988;25:350-355.
19. Burgess DE, Knoblock KF, Daughtery T, et al: Cytotoxic and hemolytic effect of *Tritrichomonas foetus* on mammalian cells. Infect Immun 1990;58:3627-3632.
20. BonDurant RH: Venereal diseases of cattle: natural history, diagnosis, and the role of vaccines in their control. Vet Clin North Am Food Anim Pract 2005;21:383-408.
21. Skirrow SZ, BonDurant RH: Bovine trichomoniasis. Vet Bull 1988;58:592-603.
22. Goodger WJ, Skirrow SZ: Epidemiologic and economic analysis of an unusually long epizootic of trichomoniasis in a large California dairy herd. J Am Vet Med Assoc 1986;189:772-776.
23. Kittell DR, Campero C, Van Hoosen KA, et al: Comparison of diagnostic methods for detection of active infection with *Tritrichomonas foetus* in beef heifers. J Am Vet Med Assoc 1998;213:519-522.
24. Bryan LA, Campbell JR, Gajadhar AA: Effects of temperature on the survival of *Tritrichomonas foetus* in transport, Diamond's and InPouch TF media. Vet Rec 1999;144:227-232.
25. Clavijo A, Erol E, Sneed L, et al: The influence of temperature and simulated transport conditions of diagnostic samples on real-time polymerase chain reaction for the detection of *Tritrichomonas foetus* DNA. J Vet Diagn Invest 2011;23:982-985.
26. Mukhufhi N, Irons PC, Michel A, et al: Evaluation of a PCR test for the diagnosis of *Tritrichomonas foetus* infection in bulls: effects of sample collection method, storage and transport medium on the test. Theriogenology 2003;60:1269-1278.

27. Davidson JM, Ondrak JD, Anderson AA, et al: Evaluation of effects of high incubation temperatures on results of protozoal culture and real-time PCR testing for *Tritrichomonas foetus* inoculated in a commercially available self-contained culture media system. J Am Vet Med Assoc 2011;239:1589-1593.
28. Skirrow SZ, BonDurant RH, Farley J, et al: Efficacy of ipronidazole against trichomoniasis in beef bulls. J Am Vet Med Assoc 1985;187:405-407.
29. Kvasnicka WG, Hanks D, Huang JC, et al: Clinical evaluation of the efficacy of inoculating cattle with a vaccine containing *Tritrichomonas foetus*. Am J Vet Res 1992;53:2023-2027.

Trichomoniasis in the bull: a review

Andrew S. Lovelady
College of Veterinary Medicine, Auburn University, Auburn, AL

Abstract

Trichomoniasis is a venereal disease of cattle of great economic impact on the cattle industry. The infected bull is the major vector for the causative organism *Tritrichomonas foetus*. An infected bull will be asymptomatic and may become infected for life after exposure. The organism is spread to the cow herd via coitus and the cow suffers the identifiable consequences of reproductive failure. There is currently no treatment for trichomoniasis in the United States. This paper will review trichomoniasis and the sexually active breeding bull including disease history, parasite morphology and life cycle, pathogenesis, prevalence, diagnostic techniques, and prevention and control strategies.

Keywords: Trichomoniasis, *Tritrichomonas foetus*, bovine, bull

Introduction

Trichomoniasis is a true venereal disease of cattle caused by the extracellular, flagellated protozoan *Tritrichomonas foetus*. Infected bulls efficiently transmit the organism to a female during coitus despite the fact they are asymptomatic carriers of *Tritrichomonas foetus*.[1] Infected females may develop vaginitis, cervicitis, or endometritis and can exhibit infertility, embryonic death, abortion, fetal maceration or pyometra.[2-5] Trichomoniasis seriously impairs the reproductive efficiency of natural service operations imposing a costly impact on the cattle industry.[6] Preventive management is the focus of disease management because no legal form of treatment exists in the United States (U.S.).

The following review of the literature on trichomoniasis most specifically pertains to the breeding bull. This compilation will review disease history, parasite morphology and life cycle, pathogenesis, prevalence, diagnostic techniques, and prevention and control strategies.

History

The venereal protozoan *T. foetus* is thought to have been originally described in France in 1888. Unfortunately, the discovery of this organism coincided with the discovery of brucellosis in 1897. Little interest was given to *Tritrichomonas foetus* until the 1920's when the Germans resumed research.[7] In 1932, Emerson described the first case of trichomoniasis in the U. S. in the state of Pennsylvania.[8] Since that time, trichomoniasis has been reported throughout the U. S. and around the world. Currently, the importance of this disease has initiated regulations for interstate, and often intrastate, movement of bulls for majority of the continental U. S.

Morphology and life cycle

Tritrichomonas means "three-haired single-celled protozoan", which accurately depicts some of the morphological characteristics of the organism as described by Taylor et al.[9] *Tritrichomonas foetus* is a pyriform-shaped protozoan with a rounded anterior end and a pointed posterior end. Its size can vary from 10 to 25 μm in length and 5 to 10 μm in width. *Tritrichomonas foetus* has a single nucleus and four flagella. Three of the flagella are located on the anterior end, while the fourth extends backwards as an extension of the undulating membrane. The undulating membrane is located on one side of the organism and has three to five waves, giving the organism a characteristic vibrating movement. This motile life stage is referred to as a trophozoite. Trophozoites undergo asexual reproduction by linear binary fission.

Trichomonads are described as having the ability to exhibit trophozoite, cyst, and pseudocyst stages across individual species.[10] The term cyst describes an invagination of external organelles to form a compact, round, non-motile life stage with a change in the exterior integrity of the cell plasma membrane to act as a protective barrier. The pseudocyst stage exhibits invagination of external organelles but does not change the composition of the plasma membrane, thus the "pseudo" designation.

Invaginated forms are often considered to be a degenerative life form in response to a stressful environment. However, *Tritrichomonas foetus* exhibits pseudocyst formation and reversal to trophozoite formation when favorable conditions return.[11] *Tritrichomonas foetus* also has the ability to undergo reproduction by multiple binary fission, a budding or "shizongony-like" division, while in this pseudocystic stage.[12] A recent report indicates that this pseudocyst form actually appears more commonly than the trophozoite stage in preputial secretions from naturally infected bulls.[13] The exact role of the pseudocyst form for *Tritrichomonas foetus* remains unclear at this time, but the fecundity of this life stage leads many to believe it may be a part of the normal life cycle.

Pathogenesis

Tritrichomonas foetus is an obligate, extracellular parasite of the bovine reproductive tract and prefers a microaerophilic to anaerobic environment. The preputial cavity of the bull provides an ideal environment where it localizes in the smegma or secretions of the epithelial lining of a bull's penis and prepuce, and may invade the external urethral orifice.[14] The organism does not invade the epithelium[15] nor typically invoke an effective immune response in the bull.[15,16] *Tritrichomonas foetus* causes no penile or preputial pathology and does not affect semen quality or libido.[15,17] Therefore, an infected bull acts only as an asymptomatic carrier. Disease transmission occurs when an infected bull breeds a non-infected cow, or a non-infected bull breeds an infected female.

The period for which a bull remains infected is a subject of much debate. Transient infections and a chronic carrier state are two popular theories regarding length of infection. The carrier state is apparently related to the depth of the preputial and penile epithelial crypts. Traditional thought is that older bulls have deeper epithelial crypts which provide the appropriate microaerophilic environment required for establishment of chronic infections.[15,18-20] However, a recent study of the histology of the penile and preputial epidermal surface of the bull argues against the existence of preputial crypts and suggests no difference in the depth of preputial skin folds between young and mature bulls.[21] The chronic carrier state associated with many *Tritrichomonas foetus* infections in bulls rarely clears regardless of time. Details regarding the complete pathology of chronic infection within the mature bull remain unknown.

Tritrichomonas foetus infections in bulls less than three to four years of age are more likely to be transient. Younger bulls may only transmit disease if sexual contact with a non-infected cow occurs within minutes to days following breeding of an infected cow.[15] Some studies indicate that clearance in a young bull is possible within minutes after breeding an infected cow.[22,23] Therefore, transmission of *T. foetus* by a young bull is more a passive, mechanical transmission, as compared to transmission associated with a chronically infected older bull. However, any bull exposed to *T. foetus* in a natural breeding situation is capable of becoming chronically infected, regardless of age.

Prevalence

The prevalence of trichomoniasis has been estimated for different areas of North America and other regions of the world. In 1964 a 7.5 % prevalence was reported in western range bulls.[24] More recent studies from Florida,[25] Oklahoma,[26] and California[27] found prevalence rates of 7.3, 7.8 and 4.1 %, respectively. An epidemiological survey in Florida between 1997 and 1999 found a 6% prevalence of *T. foetus* infected bulls.[28] A 6 % prevalence rate was reported for bulls in Saskatchewan, Canada.[29] In the North Western Cape Province, Western Transvaal, and the Orange Free State in South Africa a prevalence of 7% was determined.[30] All reports are considered to be estimates because some surveys sampled bulls from sale barns or abattoirs and others sampled bulls from randomly selected natural service beef herds.

Diagnostic technique

Sampling technique

Sampling a bull for trichomoniasis involves attempting to recover organisms from the preputial cavity and transporting the sample to a laboratory for positive identification of organisms. Sampling techniques utilized for obtaining diagnostic specimens in the bull include: 1) a swab technique;[31] 2) a dry pipette technique;[15,32] 3) a wet pipette technique;[33] and 4) the douche technique.[33] The dry pipette technique is one of the most common sampling methods in the U. S., while the douche method is the preferred technique in Europe.[32] Regardless of technique used, it is generally recommended that bulls be sexually rested one to two weeks before testing. Because breeding mechanically removes many of the organisms from a bull's penis and prepuce, sexual rest may allow for organismal replication and allow for a greater chance of recovery during sampling. Samples can be submitted for microscopic evaluation or molecular-based evaluation.

Sample analysis

Direct microscopic examination of specimens for *Tritrichomonas foetus* can be diagnostic, but a far more sensitive method for the detection of *T. foetus* is *in vitro* culture of preputial smegma in a selective nutrient medium for up to one week.[34,35] *In vitro* culture allows the proliferation of *T. foetus* to more readily detectable levels. All cultures containing organisms resembling *T. foetus* should be confirmed with appropriate molecular-based assays to avoid false-positive results due to fecal trichomonad contamination of culture media[36-38]. *In vitro* cultivation using either Diamond's medium or the InPouch™ TF is currently the most common method used to diagnose *T. foetus* in the U.S.

Alternatively, samples may be submitted directly for molecular-based evaluation. At present, polymerase chain reaction (PCR) is the test of choice for molecular based testing.[39] Improved methods[40-42] have allowed PCR to become an important diagnostic tool for trichomoniasis. In contrast to *in vitro* culture, PCR can be performed in a matter of hours offering a more rapid test result.

A single sample evaluated with either technique may yield inconclusive results, as false negatives are common. Sensitivity and specificity of both culture and PCR are maximized with sequential testing, sampling the bull at weekly intervals and repeating the same test. Positive bulls are more accurately identified when samples are tested in parallel, performing cultures and confirming with PCR on a single sample.[42] To maximize sampling technique, personal recommendation is to submit three samples at one week intervals for parallel testing. A bull should not be considered negative until three negative test results have been achieved.

Prevention and control strategies

Historically, the most successful treatment for bulls with trichomoniasis utilized systemic treatment with nitromidazole derivatives.[7] However, the use of nitromidazole derivatives is now illegal in food-producing animals in the U.S. because of their mutagenic and carcinogenic properties, and no alternative treatments exist. Therefore, bovine trichomoniasis must be prevented or controlled.

Prevention

Recommendations to avoid introducing trichomoniasis into a herd include:

1. Avoid grazing cattle on public lands to reduce exposure through coitus with other *T. foetus* infected animals.[43]
2. Utilize artificial insemination when possible.[15]
3. Use a 60-90 day breeding season. Cull all cows and heifers that are not pregnant after the breeding season. A long breeding season not only allows propagation of *T. foetus*, but may also hide production losses due to reduced weaning weights because of delayed conception.[44]
4. Control animal movement into a herd by maintaining good fences.
5. Purchase virgin bulls and heifers as replacements. Buying older bulls and cows as replacements greatly increases the chance of purchasing a *Tritrichomonas foetus*-infected animal.[45]

6. Test all bulls for *T. foetus* at least once before introducing them into a new herd.[15] The test should be performed after two weeks of sexual rest. Ideally, a bull should have three negative tests at weekly intervals.
7. Maintain as young a bull battery as possible. Older bulls are much more likely to be chronically infected with *T. foetus*.[14,46]
8. Breed purchased cows and heifers in a separate herd. Cull all the cows and heifers that are not pregnant after the breeding season. Ideally, continue to keep the pregnant animals segregated from the rest of the herd through the next breeding season.[33]

Control

Recommendations for control of trichomoniasis in an infected herd include:

1. Test and cull all infected bulls. Infected bulls should be sold for slaughter only.
2. Decrease the number of bulls per breeding unit. Single-sire herds offer the lowest exposure potential. However, single-sire units may not always be practical.
3. Reduce the average age of the bull herd.
4. Only purchase bulls from herds known to be free of *T. foetus*. All purchased bulls must pass a breeding soundness evaluation and have a least one negative *T. foetus* culture before being allowed into the herd. The test should be performed after two weeks of sexual rest. Ideally, three negative test results at weekly intervals would be obtained.
5. Utilize artificial insemination when possible.[15]
6. Reduce the breeding season to 90-120 days. Pregnancy exams should be performed 45-60 days after the breeding season. Open cows and heifers should be culled. If there are too many open cows for culling to be economically feasible, then these animals should at least be separated into a high-risk herd.
7. Culture all pyometras diagnosed in cows or heifers during pregnancy examinations.
8. Submit all aborted fetuses and placental tissue to a diagnostic laboratory.
9. Vaccinate all breeding age females against trichomoniasis. Vaccination does not offer complete protection, but it does reduce the duration of infection therefore mitigating the reproductive wastage caused by *T. foetus*.[47-50]

References

1. Rae DO, Crews JE: *Tritrichomonas foetus*. Vet Clin North Am Food Anim Pract 2006;22:595-611.
2. Fitzgerald PR: Bovine trichomoniasis. Vet Clin North Am Food Anim Pract 1986;2:277-282.
3. Mickelsen WD, Paisley LG, Anderson PB: Survey of the prevalence and types of infertility in beef cows and heifers. J Am Vet Med Assoc 1986;189:51-54.
4. Parsonson IM, Clark BL, Dufty JH: Early pathogenesis and pathology of *Tritrichomonas foetus* infection in virgin heifers. J Comp Pathol 1976;86:59-66.
5. Clark BL, Dufty JH, Parsonson IM: The effect of *Tritrichomonas foetus* on calving rates in beef cattle. Aust Vet J 1983;60:71-74.
6. Rae DO: Impact of trichomoniasis on the cow-calf producer's profitability. J Am Vet Med Assoc. 1989;194:771-775.
7. Roberts SJ: Infectious diseases causing infertility in cows. In: Veterinary obstetrics and genital diseases. 3rd ed. Ithaca(NY): Published by the author; 1986. p. 447-555.
8. Emmerson M: Trichomoniasis in cattle. J Am Vet Med Assoc 1932;34:636-640.
9. Taylor MA, Marshall RN, Stack M: Morphological differentiation of *Tritrichomonas foetus* from other protozoa of the bovine reproductive tract. Brit Vet J 1994;150:73-80.
10. Pereira-Nevesa A, Ribeirob KC, Benchimol M: Pseudocysts in trichomonads-new insights. Protist 2003;154:313-329.
11. Granger BL, Warwood SJ, Benchimol M, et al: Transient invagination of flagella by *Tritrichomonas foetus*. Parasitol Res 2000;86:699-709.
12. Pereira-Nevesa A, Benchimol M: *Tritrichomonas foetus*: budding from multinucleated pseudocysts. Protist 2009;160:536-551.
13. Pereira-Nevesa A, Campero CM, Martinez A, et al: Identification of *Tritrichomonas foetus* pseudocysts in fresh preputial secretion from bulls. Vet Parasitol 2011;175:1-8.
14. Parsonson IM, Clark BL, Dufty JH: The pathogenesis of *Tritrichomonas foetus* infection in the bull. Aust Vet J 1974;50:421-423.

15. Peter D: Bovine venereal diseases. In: Youngquist RS, editor. Current therapy in large animal theriogenology. Philadelphia: W.B. Saunders; 1997. p.355-363.
16. Cobo ER, Corbeil LB, Gershwin LJ, et al: Preputial cellular and antibody responses of bulls vaccinated and/or challenged with *Tritrichomonas foetus*. Vaccine 2009;28:361-370.
17. Anderson ML, Barr BC, Conrad PA: Protozoal causes of reproductive failure in domestic ruminants. Vet Clin North Am Food Anim Pract 1994;10:439-461.
18. BonDurant RH: Pathogenesis, diagnosis and management of trichomoniasis in cattle. Vet Clin North Am Food Anim Pract 1997;13:345-361.
19. Honigberg BN: Trichomonads of veterinary importance: *Tritrichomonas foetus*. In: Kreier JP, editor. Parasitic protozoa. New York: Academic Press; 1978. p.207-273.
20. BonDurant RH, Honigberg BN: Trichomonads of veterinary importance. In: Kreier JP, editor. Parasitic protozoa. 2nd ed. San Diego: Academic Press; 1994. p.111-188.
21. Strickland LG, Wolfe DF, Carson RL, et al: Surface architectural anatomy of the penile and preputial epithelia of bulls [abstract]. Clin Therio 2011;3:362
22. Morgan BB: Studies on the trichomonad carrier-cow problem. J Anim Sci 1944;3:437.
23. Clark BL, Dufty JH, Parsonson RD, et al: Studies on the transmission of *Tritrichomonas foetus*. Aust Vet J 1977;53:170-172.
24. Johnson AE: Incidence and diagnosis of trichomoniasis in western beef bulls. J Am Vet Med Assoc 1964;145:1007-1010.
25. Abbitt B, Meyerholz GW: *Trichomonas foetus* infection of range bulls in South Florida. Vet Med Small Anim Clin 1979;74:1339-1342.
26. Wilson SK, Kocan AA, Gaudy ET, et al: The prevalence of trichomoniasis in Oklahoma beef bulls. Bov Pract 1979;14:109-110.
27. BonDurant RH, Anderson ML, Blanchard P, et al: Prevalence of trichomoniasis among California beef herds. J Am Vet Med Assoc 1990;196:1590-1593.
28. Rae DO, Crews JE, Greiner EC, et al: Epidemiology of *Tritrichomonas foetus* in beef bull populations in Florida. Theriogenology 2004;61:605-618.
29. Riley DE, Wagner B, Polley L, et al: PCR-based study of conserved and variable DNA sequences of Tritrichomonas foetus isolates from Saskatchewan, Canada. J Clin Microbiol 1995;33:1308-1313.
30. Erasmus JA, De Wet JAL, Van der Merwe HE, et al: Bovine trichomoniasis in the North Western Cape Province, Western Transvaal, and the Orange Free State. J S Afr Vet Assoc 1989;60:51-52.
31. Morgan BB: Bovine trichomoniasis. Minneapolis: Burgess Publishing Company; 1946.
32. Schönmann MJ, BonDurant RH, Gardner IA, et al: Comparison of sampling and culture methods for the diagnosis of *Tritrichomonas foetus* infection in bulls. Vet Rec 1994;134:620-622.
33. Kimsey PB: Bovine trichomoniasis. In: Morrow DA, editor. Current therapy in theriogenology. 2nd ed. Philadelphia: W.B. Saunders; 1986. p.275-279.
34. Johnson AE: The diagnosis of trichomoniasis in the bull. Proc US Livest Sanit Assoc 1965;69:183-189.
35. Tedesco LF, Errico F, Baglivi LP: Diagnosis of *Tritrichomonas foetus* infection in bulls using two sampling methods and a transport medium. Aust Vet J 1979;55:322-324.
36. BonDurant RH, Gajadhar A, Campero CM, et al: Preliminary characterization of a *Tritrichomonas foetus*-like protozoan isolated from preputial smegma of virgin bulls. Bov Pract 1999;33:124-127.
37. Campero CM, Rodriguez Dubra C, Bolondi A, et al: Two-step (culture and PCR) diagnostic approach for differentiation of non-*T. foetus* trichomonads from genitalia of virgin beef bulls in Argentina. Vet Parasitol 2003;112:167-175.
38. Cobo ER, Campero CM, Mariante RM, et al: Ultrastructural study of a tetratrichomonad species isolated from prepucial smegma of virgin bulls. Vet Parasitol 2003;117:195-211.
39. Felleisen RSJ, Lambelet N, Bachmann P, et al: Detection of *Tritrichomonas foetus* by PCR and DNA enzyme immunoassay based on rRNA gene unit sequences. J Clin Microbiol 1998;36:513-519.
40. Ho MSY, Conrad PA, Conrad PJ, et al: Detection of bovine trichomoniasis with a specific DNA probe and PCR amplification system. J Clin Microbiol 1994;32:98-104.
41. McMillen L, Lew AE: Improved detection of *Tritrichomonas foetus* in bovine diagnostic specimens using a novel probe-based real time PCR assay. Vet Parasitol 2006;141:204-215.
42. Cobo ER, Favetto PH, Lane VM, et al: Sensitivity and specificity of culture and PCR of smegma samples of bulls experimentally infected with *Tritrichomonas foetus*. Theriogenology. 2007;68:853-860.
43. Gay JM, Ebel ED, Kearley WP: Commingled grazing as a risk factor for trichomonosis in beef herds. J Am Vet Med Assoc 1996;209:643-646.
44. Kvasnicka WG, Hall MR, Hanks DR: Bovine trichomoniasis. In: Howard JL, editor. Current veterinary therapy 4: food animal practice. Philadelphia: W.B. Saunders; 1999. p.420-425.
45. Skirrow S: Identification of trichomonad-carrier cows. J Am Vet Med Assoc 1987;191:553-554.
46. Rae DO, Chenoweth PJ, Genho PC, et al: Prevalence of *Tritrichomonas fetus* in a bull population and effect on production in a large cow-calf enterprise. J Am Vet Med Assoc 1999;214:1051-1055.

47. Hall MR, Kvasnicka WG, Hanks D, et al: Improved control of trichomoniasis with *Trichomonas foetus* vaccine. Agri Pract 1993;14:29-34.
48. Kvasnicka WG, Hanks D, Huang J, et al: Clinical evaluation of the efficacy of inoculating cattle with a vaccine containing *Tritrichomonas foetus*. Am J Vet Res 1992;53:2023-2027.
49. Schnackel JA, Wallace BL, Kvasnicka WG, et al: *Trichomonas foetus* vaccine immunogenicity trial. Agri Pract 1989;10:11-14.
50. Gault RA, Kvasnicka WG, Hanks D, et al: Specific antibodies in serum and vaginal mucus of heifers inoculated with a vaccine containing *Tritrichomonas foetus*. Am J Vet Res 1995;56:454-9.

Cattle physiology and the CO-Synch + CIDR synchronization program

M.L. Day,[a] G. Allen Bridges[b]
[a]Department of Animal Sciences, The Ohio State University, Columbus, OH; [b]North Central Research and Outreach Center, University of Minnesota, Grand Rapids, MN

Introduction

The most commonly used approaches to timed-artificial insemination (AI) in beef cattle in the USA are based upon the CO-Synch program.[1] In the USA, three hormones are available to synchronize cows; progesterone (usually a vaginal insert; CIDR), prostaglandin $F_{2\alpha}$ (PGF; or its analog) and gonadotropin releasing hormone (GnRH). The original CO-Synch program consisted of an initial GnRH treatment, PGF seven days later to induce luteolysis, and a second GnRH treatment 48 h after PGF to induce ovulation for timed-AI. The timing of the second injection of GnRH determines the length of "proestrus", or the interval between the initiation of regression of the corpus luteum (CL) and the luteinizing hormone (LH) surge. A CIDR is usually inserted into beef females between the initial GnRH and the PGF treatment, resulting in a CO-Synch + CIDR program. Each exogenous hormone used in this program has specific actions and the efficacy and accuracy of these actions are crucial for synchronization. The first GnRH treatment is used to induce ovulation and reset follicular growth. In other words, approximately one to two days after GnRH a new follicular wave should be initiated in a majority of cows.[2-5] The efficacy of the initial GnRH, however, varies among animal class and stage of the estrous cycle.[6-10] The second GnRH will induce an LH surge and subsequent ovulation of the dominant follicle that results from the new wave induced by the first GnRH. Luteolysis is induced with PGF between 54 and 72 hours before the second GnRH treatment. Timed-AI is performed coincident with the second administration of GnRH. One important concern is the proportion of cows that are induced to ovulate follicles that are smaller than typical diameter with the second GnRH administration and the fact these animals are less likely to become pregnant to timed-AI.[11,12]

The influence of ovulatory follicle maturity on fertility in beef cattle has been investigated.[12-15] One hypothesis was that diameter of ovulatory follicles was the most appropriate indicator of follicle "maturity" and that cows induced to ovulate small follicles would have lesser fertility compared to those induced to ovulate larger follicles. Within each of three experiments (Table 1[13,14]) this hypothesis was supported, but as data from multiple experiments accumulated, the relationship of follicle diameter to pregnancy rate appeared inconsistent. Across experiments, the more consistent predictor of pregnancy rate appeared to be duration of proestrus (interval from initiation of CL regression with PGF to the LH surge; Table 1). Based on the relationship between length of proestrus and conception rate, an additional experiment (Table 1;[15]) was performed to hold follicle diameter constant and only vary length of proestrus. It was demonstrated that at a constant ovulatory follicle diameter, length of proestrus had a substantial influence on conception rate. Taken together, data from this series of studies suggested a strong positive relationship of duration of proestrus with follicle maturity and fertility and suggested that diameter of the ovulatory follicle, in itself, was not a consistent predictor of follicle maturity. The effect of ovulatory follicle diameter at GnRH-induced ovulation or at spontaneous ovulation on conception rate has also been evaluated.[12,16] It was reported that diameter of the ovulatory follicle influenced conception rate after detection of estrus in heifers, but not in postpartum cows. In postpartum cows that did not exhibit estrus, diameter of the ovulatory follicle was positively associated with conception rate when ovulation was induced with GnRH. Thus, if a 'complete' spontaneous proestrus occurred in cows (confirmed by exhibition of estrus), diameter did not impact fertility, but, diameter of the ovulatory follicle when ovulation was induced with GnRH did influence conception rate; at a constant duration of proestrus. Since findings suggested that maturity of the ovulatory follicle and probability of conception was perhaps defined by length of proestrus, we applied this knowledge towards optimizing the existing CO-Synch + CIDR program.

Keywords: CO-Synch + CIDR, estrus synchronization, follicle growth, follicle age, proestrus

Table 1. Conception rate, diameter and age of the ovulatory follicle, length of proestrus, and number of cows in a series of experiments investigating the effect of follicle maturity on fertility.

Conception Rate (%)[a]	Follicle diameter at Ovulation (mm)[b]	Duration of Proestrus (days)[c]	n	Experiment
4	11.1 ± 0.2	1.0 ± 0.1	45	Mussard et al., 2003a[e]
8	11.1 ± 0.2	1.0 ± 0.1	12	Mussard et al., 2003b[f]
10	12.6 ± 0.2	1.25	10	Bridges et al., 2010[g]
57	13.6 ± 0.2	2.2 ± 0.1	54	Mussard et al., 2003a[e]
67	13.7 ± 0.2	2.0 ± 0.1	12	Mussard et al., 2003b[f]
71	12.9 ± 0.2	2.25	28	Bridges et al., 2010[g]
76	10.7 ± 0.1	3.3 ± 0.1	29	Mussard et al., 2007[d]
100	12.0 ± 0.3	4.7 ± 0.2	24	Mussard et al., 2007[d]

[a] Percentage of animals determined to be pregnant following insemination. Pregnancy determination was conducted via ultrasonography at approximately 30 days post-insemination.
[b] Diameter of the largest ovulatory follicle as determined by ultrasonography conducted either at GnRH administration or estrus.
[c] Interval from $PGF_{2\alpha}$ until GnRH administration.
[d] Cows were either induced with GnRH to ovulate a small (~11 mm) follicle or allowed to spontaneously exhibit estrus. Cows were inseminated 12 hours following estrus or GnRH.
[e] Cows were induced to ovulate either a small (~11 mm) or large (~13 mm) ovarian follicle with GnRH. Animals were inseminated 12 h following GnRH administration.
[f] Cows were induced to ovulate either a small (~11 mm) or large (~13 mm) ovarian follicle with GnRH. Embryo from non-treated cows were then transferred 7 days after GnRH.
[g] Cows were induced to ovulate an ovarian follicle of similar diameter with GnRH either 1.25 or 2.25 days following $PGF_{2\alpha}$ administration. Animals were inseminated 12 h following GnRH administration. Includes only cows with a luteal phase of normal length.

Lengthening proestrus in the CO-Synch + CIDR program

The length of proestrus with the traditional 7-day CO-Synch + CIDR program was varied from 50 to 66 hours in mature cows without influencing timed-AI pregnancy rate, but in younger cows (≤ 3 years of age), greatest pregnancy rates were achieved with timed-AI at 56 hours.[17] Others[18] have reported that timed-AI pregnancy rates were greater when proestrus was 66 than 54 hours. In practice, the second GnRH is given and timed-AI is performed in most herds between 54 and 66 hours after PGF. We hypothesized that if the CO-Synch + CIDR synchronization approach could be modified in a manner in which we could increase the interval from PGF and CIDR removal to the second GnRH and timed-AI, that timed-AI pregnancy rate would increase. This end was achieved through development of the 5-d CO-Synch + CIDR program[19] and is the focus of the companion paper (Bridges and Day, 2013) to this review. The remainder of this review focuses on the physiological effects of this change in the program and potential mechanisms for the increase in timed-AI pregnancy rate that is achieved.

Hormonal changes with a lengthened proestrus

Proestrus starts with removal of progesterone sources (a CL, a CIDR or both) and ends with either a spontaneous or GnRH- induced LH surge. Concentrations of progesterone decline rapidly and are sustained at basal concentrations throughout proestrus, setting off a series of crucial hormonal changes that precede ovulation. An almost immediate response to declining progesterone concentrations is an increase in the frequency of LH pulses. Frequency of release of LH from the anterior pituitary is

primarily regulated by progesterone and the negative association of progesterone concentration and frequency of LH pulses has been well established.[20] Proestrus is characterized by LH pulses at an increasing frequency as proestrus progresses and the LH surge approaches.[21] Pulsatile LH secretion is the primary factor that drives the final development of preovulatory follicles. During a spontaneous proestrus, growth of the preovulatory follicle and production of estradiol by granulosa cells in the follicle increases as proestrus progresses. We have compared preovulatory estradiol and post-ovulatory progesterone concentrations, and the magnitude of the LH surge between female cattle experiencing either a long (54 hr; LPE) or short (30 hr; SPE) proestrus.[15] Ovulatory follicle size and magnitude of the GnRH-induced LH surge did not differ, but there tended to be a greater incidence of short estrous cycles and lesser progesterone concentrations during the subsequent estrous cycle in the SPE than LPE treatment. The most striking difference between treatments was that concentrations of estradiol were greater in the LPE than SPE treatment during the 38 hours preceding GnRH (Figure 1). Consistent with this observation, cows that received the 5-d vs. the 7-d CO-Synch + CIDR program ovulated follicles of similar diameter that tended to produce greater peak estradiol concentrations.[22] A logical explanation for this difference in estradiol concentrations is the extended period of stimulation by high frequency LH pulses. However, an additional factor that we think may also contribute to enhanced systemic estradiol with a 5-d vs. 7-d program is that removal of progesterone restraint of LH secretion occurs earlier relative to follicular wave emergence. With a 5-d, as compared to a 7-d program, follicles resulting from the new wave initiated after the first GnRH injection would be approximately three to four days post-emergence, vs. five to six days from emergence, respectively, at PGF and CIDR removal. It has been reported that growing dominant follicles, four days after emergence, have increased intra-follicular estradiol concentrations and capacity to produce estradiol in vitro than non-atretic dominant follicles at a time later in the follicular wave.[23] Furthermore, it has been demonstrated that concentrations of estradiol in the caudal vena cava were greater[24] at approximately three days after emergence of the first wave dominant follicle as compared to later in the lifespan of this follicle. Hence, extending proestrus and removing progesterone at a time when steroidogenic capacity of dominant follicles is optimal may both contribute to greater peak concentrations and/or an extended period of elevated estradiol during proestrus. The concentrations of estradiol present during the preovulatory period in cattle is increasingly recognized as a key factor that influences fertility.[25-29] We have concluded that a key impact of increased length of proestrus is to escalate preovulatory concentrations of estradiol in response to a longer period of LH stimulation and have demonstrated greater estradiol concentrations during proestrus and an increased timed-AI pregnancy rate in the 5-d as compared to the 7-d CO-Synch + CIDR program.

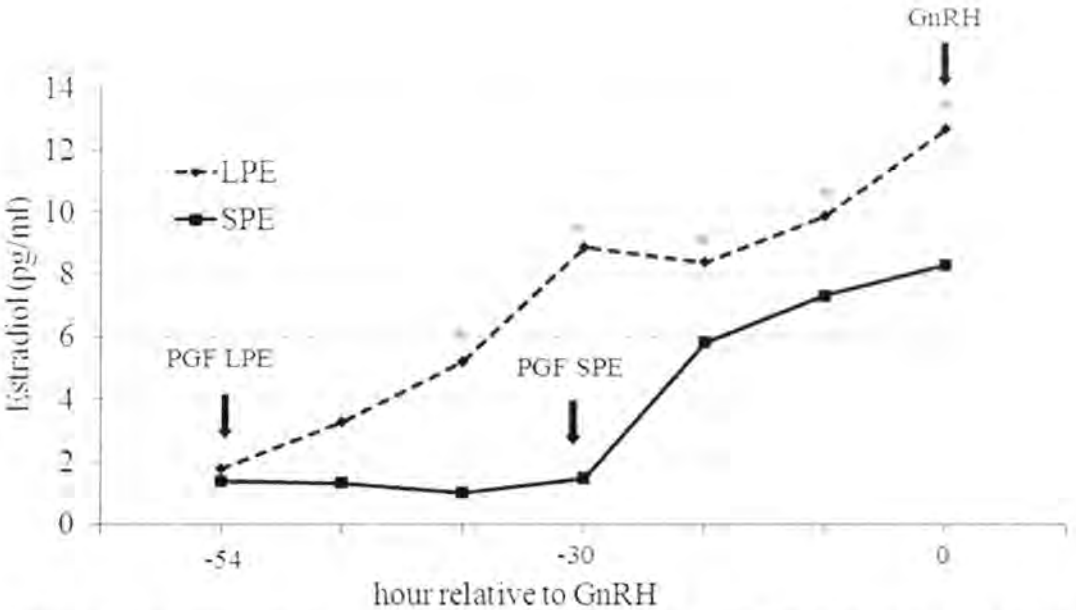


Figure 1. Concentrations of estradiol in cows that experienced either a long (LPE) or short (SPE) proestrus. Adapted from Bridges et al., 2010

Follicular growth and length of synchronization program

It has been demonstrated that the 7 day CO-Synch program results in a proportion of cows that ovulate follicles of smaller than typical diameter at timed-AI, which results in decreased fertility in these animals.[11,12] We and others have presumed that the smaller follicles at the time of synchronized ovulation are the result of spontaneous atresia of follicles and initiation of a new wave of follicular development during the latter stages of the interval between the initial GnRH treatment and PGF in females that do not respond to the first GnRH treatment. It has been reported that this is one of a variety of factors contribute to the variation in diameter of follicles at the synchronized ovulation.[8,9] Reducing the length of CIDR treatment from seven to five days would be expected to reduce the likelihood that this pattern of follicular growth would occur, and result in greater estradiol concentrations during proestrus in these females. Failure to ovulate to the first GnRH resulted in reduced preovulatory estradiol concentrations and progesterone concentrations during the subsequent luteal phase in the 7-d but not in the 5-d CO-Synch + CIDR program.[22] In a recent preliminary experiment (Bridges GA, unpublished) pregnancy rate to timed-AI in cows not responding to the first GnRH was similar to, or greater than that of cows that did respond to the initial GnRH. Further research regarding this preliminary finding, and whether a portion of the benefit of the 5-day program is the result of normal fertility in those females not responding to the initial GnRH is necessary.

Age of follicles at induced ovulation

In the 5-d CO-Synch + CIDR program, the interval from follicle wave emergence at one to two days after the first GnRH, to induction of ovulation with the second GnRH (day 8) is six to seven days. In the 7-d program, this interval is eight to nine days. We refer to this interval as follicle age, and due to the design of the 5-d program, younger follicles are induced to ovulate with the second GnRH as compared to the 7-d program. Variation in follicle age at ovulation does exist in spontaneously ovulating cows. For example, in spontaneously ovulating dairy cows that have either two or three waves of follicular growth during their estrous cycle, the interval from follicle emergence to estrus (age of the follicle) is greater by approximately three days in cows with two follicular waves[30] and pregnancy rate to AI is lower when compared to cows with three follicular waves during the estrous cycle.[31] Use of a five day interval between GnRH and PGF increased pregnancy rate in lactating dairy cows[32] and Cerri et al[33] demonstrated

a greater proportion of good quality embryos collected from lactating dairy cows that were induced to ovulate younger follicles; within the range normally observed in spontaneously ovulating females. The cumulative interpretation of reports in lactating dairy cows suggests that age of the follicle is a significant source of variation in fertility. We have recently completed two experiments to directly address the effect of age of the ovulatory follicle on fertility in cattle and tested the hypothesis that conception rate to AI after ovulation of a younger follicle would be greater in beef heifers after spontaneous ovulation and in postpartum beef cows after either a spontaneous or GnRH-induced ovulation.

In the first experiment in heifers (n = 280, Montana and Ohio heifers), luteal regression was induced with PGF either two (young follicle = YF) or six (mature follicle = MF) d after emergence of a new follicular wave and heifers were AI 12 hours after expression of estrus.[34] As expected, the interval from PGF to estrus was greater in the YF than MF group with some variation in this interval between locations (Table 2). Age of follicles at AI was greater by approximately three days in the MF group, and diameter of the ovulatory follicle was marginally greater in the MF than YF heifers. However, conception rate to estrus-AI did not differ between groups.

Table 2. Estrous response, proestrus interval, follicle age and size at AI (Mean ± SE), and conception rate in heifers ovulating a mature (MF) or young (YF) follicle.

		n	Estrous response, %	Proestrus [d] interval, h	Follicle age [d] at AI, d	Follicle size at AI, mm	Conception rate, %
Montana	MF	53	92.5	55.8 ± 2.7[a]	8.3 ± 0.11[a]	11.0 ± 0.18[a]	63.3
	YF	75	90.7	67.4 ± 1.6[b]	4.8 ± 0.06[b]	10.4 ± 0.15[b]	64.7
Ohio	MF	77	87.0	53.7 ± 2.2[a]	8.2 ± 0.10[a]	-	64.2
	YF	75	90.7	78.5 ± 1.4[c]	5.3 ± 0.06[c]	-	69.1

[a,b,c] Values with different superscripts in the same column differ ($P < 0.01$).
[d] trt x loc ($P < 0.01$) for proestrus interval and follicle age at AI.

In postpartum cows[35] (n = 243), luteal regression was induced with PGF either 2.5 (young follicle = YF) or 6.5 (mature follicle = MF) d after emergence of a new follicular wave. Based upon the intervals to estrus in heifers,[34] cows in the MF group were AI based upon estrus detection until 72 hours after PGF with the cows not detected in estrus receiving GnRH and timed-AI at hour 72. In the YF group, estrus detection and AI was performed to hour 96, with timed-AI in the remaining cows at hour 96. Interval to estrus after PGF was approximately 24 h greater in the YF than MF treatment (Table 3). This resulted in a difference in follicle age at AI of approximately three days (MF > YF) yet diameter of the ovulatory follicle did not differ between treatments. Pregnancy rate during the synchronization period did not differ between cows in the MF and YF treatments (Table 3).

Table 3. Reproductive variables (Mean ± SE) in postpartum beef cow ovulating either a mature (MF) or young (YF) follicle.

Variable	MF	YF	*P* value
Estrous response within 72 h, %	76.6	48.3	< 0.01
Estrous response, PGF to TAI (72 vs. 96 h), %	76.6	88.6	< 0.05
Interval from PGF to estrus, h	56.7 ± 1.7	79.0 ± 0.7	< 0.01
Follicle age at AI, d	9.01 ± 0.06	5.87 ± 0.03	< 0.01
Follicle diameter at AI, mm	13.1 ± 0.2	13.0 ± 0.1	> 0.10
Follicle growth rate (PGF to AI), mm/d	1.15 ± 0.08	1.29 ± 0.04	= 0.09
Pregnancy rate, %	72.3	67.1	> 0.10

As previously described, in cattle that initiate a new follicle wave after the first GnRH, age of the ovulatory follicle for a 5-d program, by design, is approximately two days less than with a 7-d CO-Synch + CIDR program. Results of experiments by Abreu et al[34,35] suggest that age of ovulatory follicles, in itself, for females that respond to the first GnRH may not be a substantial source of variation in timed-AI pregnancy rate.

Summary

Based upon observations across a series of experiments that pregnancy rate to timed-AI was positively related to length of proestrus, the traditional 7-d CO-Synch + CIDR program was modified to allow an increased interval from PGF/CIDR removal to GnRH/timed-AI; resulting in the 5-d CO-Synch + CIDR program. This modification has been demonstrated to increase timed-AI pregnancy rates relative to the traditional approach. The impact of this modification on preovulatory estradiol concentrations, as a result of extending the period of gonadotropic stimulus provided to the follicle, initiation of proestrus at a time when ovulatory follicles are highly estrogenic and/or through reduction in the incidence of ovulation of very young follicles, are potential mechanisms for increased estradiol concentrations and enhanced fertility. Conversely, for females in these estrous control programs in which follicular growth is adequately controlled, differences in age of the ovulatory follicle may not be a significant contributor to variation in timed-AI pregnancy rate.

Acknowledgements

Appreciation is expressed to OSU-OARDC, USDA-NIFA, USDA-ARS (Miles City, MT), Select Sires Inc., Zoetis (formerly Pfizer Animal Health), and IVX Animal Health for support of various experiments presented. In addition, the authors would like to thank the numerous individuals and other laboratories that assisted with research presented herein.

References

1. Geary TW, Whittier JC: Effects of a timed insemination following synchronization of ovulation using the Ovsynch or COSynch protocol in beef cows. Prof Anim Sci 1998;14:217-220.
2. Thatcher WW, Macmillan KL, Hansen PJ, et al: Concepts for regulation of corpus luteum function by the conceptus and ovarian follicles to improve fertility. Theriogenology 1989;31:149-164.
3. Macmillan KL, Thatcher WW: Effects of an agonist of gonadotropin-releasing hormone on ovarian follicles in cattle. Biol Reprod 1991;45:883-889.
4. Twagiramungu H, Guilbault LA, Proulx JG,et al: Influence of corpus luteum and induced ovulation on ovarian follicular dynamics in postpartum cyclic cows treated with buserelin and cloprostenol. J Anim Sci 1994;72:1796-1805.
5. Twagiramungu H, Guilbault LA, Dufour JJ: Synchronization of ovarian follicular waves with a gonadotropin-releasing hormone agonist to increase the precision of estrus in cattle: a review. J Anim Sci 1995;73:3141-151.

6. Pursley JR, Mee MO, Wiltbank MC: Synchronization of ovulation in dairy cows using PGF2α and GnRH. Theriogenology 1995;44:915-923.
7. Geary TW, Downing ER, Bruemmer JE, et al: Ovarian and estrous response of suckled beef cows to the select synch estrous synchronization protocol. Prof Anim Sci 2000;16:1-5.
8. Atkins JA, Busch DC, Bader JF, et al: Gonadotropin-releasing hormone-induced ovulation and luteinizing hormone release in beef heifers: effect of day of the cycle. J Anim Sci 2008;86:83-93.
9. Atkins JA, Smith MF, Wells KJ, et al: Factors affecting pre-ovulatory follicle diameter and ovulation rate to GnRH in postpartum beef cows, Part I: Cycling cows. J Anim Sci 2010;88:2300-2310.
10. Souza AH, Cunha AP, Silva EPB, et al: Comparison of gonadorelin products in lactating dairy cows: Efficacy based on induction of ovulation of an accessory follicle and circulating luteinizing hormone profiles. Theriogenology 2009;72:271-279.
11. Lamb GC, Stevenson JS, Kesler DJ, et al: Inclusion of an intravaginal progesterone insert plus GnRH and prostaglandin F2α for ovulation control in postpartum suckled beef cows. J Anim Sci 2001;79:2253-2259.
12. Perry GA, Smith MF, Lucy MC, et al: Relationship between follicle size at insemination and pregnancy success. Proc Nat Acad Sci 2005;102:5268-5273.
13. Mussard ML, Burke CR, et al: Ovarian follicle maturity at induced ovulation influences fertility in cattle. Proc Annu Meet Soc Therio; 2003. p. 185-197.
14. Mussard ML, Burke CR, Behlke EJ, et al: Influence of premature induction of an LH surge with GnRH on ovulation, luteal function and fertility in cattle. J Anim Sci 2007;85:937-943.
15. Bridges GA, Mussard ML, Burke CR, et al: Influence of the length of proestrus on fertility and endocrine function in female cattle. Anim Reprod Sci 2010;117:208-215.
16. Perry GA, Smith MF, Roberts AJ, et al: Relationship between size of the ovulatory follicle and pregnancy success in beef heifers. J Anim Sci 2007;85:684-689.
17. Dobbins CA, Eborn DR, Tenhouse DE, et al: Insemination timing affects pregnancy rates in beef cows treated with CO-Synch protocol including an intravaginal progesterone insert. Theriogenology 2009;72:1009-1016.
18. Busch DC, Schafer DJ, Wilson DJ, et al: Timing of artificial insemination in postpartum beef cows following administration of the CO-Synch + controlled internal drug-release protocol. J Anim Sci 2008;86:1519-1525.
19. Bridges GA, Helser LA, Grum DE, et al: Decreasing the interval between GnRH and PGF2α from 7 to 5 days and lengthening proestrus increases timed-AI pregnancy rates in beef cows. Theriogenology 2008;69:843-851.
20. Kinder JE, Kojima FN, Bergfeld EGM, et al: Progestin and estrogen regulation of pulsatile LH release and development of persistent ovarian follicles in cattle. J Anim Sci 1996;74:1424-1440.
21. Imakawa K, Day ML, Zalesky DD, et al: Regulation of pulsatile LH secretion by ovarian steroids in the heifer. J Anim Sci 1986;63:162-168.
22. Bridges GA, Mussard ML, Helser LA, et al: Comparison of follicular dynamics and hormone concentrations between the 7 d and 5 d CO-Synch + CIDR program in two-year old beef cows (abstract). J Anim Sci 2009;87(Suppl 2):372.
23. Valdez KE, Cuneo SP, Gorden PJ, et al: The role of thecal androgen production in the regulation of estradiol biosynthesis by dominant bovine follicles during the first follicular wave. J Anim Sci.\ 2005;83:597-603.
24. Rhodes FM, Fitzpatrick LA, Entwistle KW, et al: Hormone concentrations in the caudal vena cava during the first ovarian follicular wave of the oestrous cycle in heifers. J Reprod Fertil 1995;104:33-39.
25. Bridges GA, Mussard ML, Pate JL, et al: Impact of preovulatory estradiol concentrations on conceptus development and uterine gene expression Anim Reprod Sci 2012;133:16-26.
26. Atkins JA, Smith MF, MacNeil MD, et al: Pregnancy establishment and maintenance in cattle. J Anim Sci 2013;91:722-733.
27. Jinks EM, Smith MF, Atkins JA, et al: Preovulatory estradiol and the establishment and maintenance of pregnancy in suckled beef cows. J Anim Sci 2013;91:1176-1185.
28. Bridges GA, Day ML, Geary TW, et al: Deficiencies in the uterine environment and failure to support embryonic development. J Anim Sci 2012;91:doi: 10.2527/jas.2012-5882.
29. Geary TW, Smith MF, MacNeil MD, et al: Influence of follicular characteristics at ovulation on early embryonic survival. J Anim Sci 2012;91:doi: 10.2527/jas.2012-5887.
30. Bleach ECL, Glencross RG, Knight PG: Association between ovarian follicle development and pregnancy rates in dairy cows undergoing a spontaneous oestrus cycle. Reproduction 2004;127:621-629.
31. Townson DH, Tsang PC, Butler WR, et al: Relationship of fertility to ovarian follicular waves before breeding in dairy cows. J Anim Sci 2002;80:1053-1058.
32. Santos JE, Narciso CD, Rivera F, et al: Effect of reducing the period of follicle dominance in a timed artificial insemination protocol on reproduction of dairy cows. J Dairy Sci 2010;93:2976-2988.
33. Cerri RL, Rutigliano HM, Chebel RC, et al: Period of dominance of the ovulatory follicle influences embryo quality in lactating dairy cows. Reproduction 2009;137:813-823.
34. Abreu FM, Cruppe LH, Maquivar M, et al: Effect of follicle age on conception rate in beef heifers (abstract). J Anim Sci 2011;89(E-Suppl 1):253.
35. Abreu FM, Cruppe LH, Roberts CA, et al: The effect of follicle age on pregnancy rate in beef cows (abstract). J Anim Sci 2011;89(E Suppl 2):78.

Applications of physiological principles to optimize timed AI pregnancy rates with the 5-d Co-Synch + CIDR protocol in cattle

G. Allen Bridges,[a] M.L. Day[b]
[a]North Central Research & Outreach Center, University of Minnesota,Grand Rapids, MN; [b]Department of Animal Sciences, The Ohio State University, Columbus, OH

Abstract

The 5-d CO-Synch + CIDR protocol was developed to compliment fundamental research that identified physiological and endocrine factors that impact the probability of pregnancy in cattle. Initial studies in beef cows demonstrated that reducing the interval from controlled internal drug release (CIDR) insertion and gonadotropin releasing hormone (GnRH) to CIDR removal from seven to five days and extending the period from CIDR removal to timed artificial insemination (AI) from 60 to 72 h increased timed-AI pregnancy rates in beef cows. Although initial studies with the 5-d approach utilized two doses of prostaglandin $F_{2\alpha}$ (PGF) given at a 12 h interval at CIDR removal, subsequent investigations in beef cows indicate that although two doses of $PGF_{2\alpha}$ are necessary, these doses can be delivered simultaneously at CIDR removal without affecting timed-AI pregnancy rates. The 5-d approach to estrous synchronization has also been identified as an effective method for facilitating AI in heifers. Although investigations are still ongoing to develop the 'optimal' approach when using the 5-d protocol in heifers, in its current form the 5-d CO-Synch + CIDR and the 5-d Select Synch + CIDR and timed-AI protocols have been demonstrated to yield increased AI pregnancy rates in beef heifers compared to the 7-d approaches and similar AI pregnancy rates to the longer 14-d CIDR protocols. In summary, the 5-d approach to estrous synchronization in cattle is an effective protocol that results in greater AI pregnancy rates than most protocols currently available.

Keywords: 5-d CO-Synch + CIDR, beef cows, heifers, estrous synchronization, timed-AI

Introduction

Through decades of research, several estrous synchronization protocols have been developed to facilitate the use of AI in cattle and capture the reproductive and production benefits that these reproductive technologies yield. Continued investigations led to the refinement of these approaches and the development of protocols that synchronize ovulation to facilitate timed-AI. In the USA however, development of new protocols is limited by the pharmaceutical products available to facilitate synchronization. Only three hormones are available to synchronize cows, progesterone (usually a vaginal insert; CIDR), PGF (or its analog) and GnRH. Therefore, the onus to scientists aiming to improve methods of ovulation synchronization is to improve the understanding of the endocrine and physiological parameters that influence reproductive competence in cattle and design new approaches of synchronization within the limitations of the pharmaceutical products available to compliment these basic scientific discoveries. It was through this mindset that the 5-d CO-Synch + CIDR protocol was developed.

The companion review to this manuscript (M.L. Day, 2013) has addressed the physiological and endocrine factors that influence the probability of conception in cattle and how these concepts led to the development of the 5-d CO-Synch + CIDR protocol. The objective of this review is to provide an overview of the reproductive responses observed when implementing the 5-d CO-Synch + CIDR protocol in cattle. Ultimately, this review will demonstrate how furthering the basic understanding of factors that influence fertility and subsequent application of these principles into an improved methodology to synchronize ovulation in cattle, the 5-d CO-Synch + CIDR protocol, resulted in an improvement in timed-AI pregnancy rates in cattle.

Application of the 5-d CO-Synch + CIDR protocol in Beef Cows

The original investigations of reducing the interval from GnRH and CIDR insertion to CIDR removal and PGF administration from seven to five days was conducted in suckled beef cows.[1] In all initial experiments, two doses of PGF were given, the first at CIDR removal and the second 12 h later.

This approach was taken due to the relatively short interval between GnRH and $PGF_{2\alpha}$ in the 5-d treatment, as it was questionable whether the accessory corpus luteum (CL) would regress in all cows with a single injection of PGF in this treatment. Our intent was to determine whether shortening this interval would increase fertility and the second PGF dose would ensure findings were not confounded by incidence of luteal regression. Likewise, to avoid compromising the experimental approach, both the 7 and 5-d treatments received two doses of PGF. Furthermore, in each experiment the initiation of protocols were offset, if necessary, so that all cows were inseminated at the same time on the same day, or during the same series of days if estrus and AI was used. This approach permitted cows to be inseminated randomly relative to treatment to avoid possible confounding effects of environment. The first experiment compared a 7- versus 5-d Select Synch + CIDR protocol and cows were inseminated following an observed estrus. Estrous response and interval to estrus did not differ between treatments. Not surprisingly, when cows were only inseminated after exhibiting estrus, when probability of conception is generally accepted as the greatest, AI conception rate nor AI pregnancy rate differed between treatments. In the second experiment, timed-AI was exclusively implemented, comparing the 7-d and the 5-d CO-Synch + CIDR protocols when timed-AI was conducted 60 h after CIDR removal in both treatments. With this common interval of proestrus (60 h), timed-AI pregnancy rates did not differ between the 7-d (52.7%, n = 112) and 5-d (56.8%, n = 111) treatments. In the final two experiments the proestrus interval (the interval from CIDR removal and PGF to GnRH administration and timed-AI) was lengthened from 60 to 72 h in the 5-d approach. Such a modification was done in an effort to maximize preovulatory estradiol concentrations, which has been demonstrated, as highlighted in the companion paper, to increase the probability of pregnancy success in cattle. With this lengthened interval of proestrus in the 5-d approach, timed-AI pregnancy rates were improved by 13.3 and 9.1 percentage points for each experiment compared to timed-AI pregnancy rates for the 7-d approach when timed-AI was performed at 60 h after CIDR removal (Table 1). This improvement in timed-AI pregnancy rates occurred in all classifications of suckled beef cows; anestrus and cyclic and primiparous and multiparous. Collective consideration of these first four experiments indicted that reducing the interval from CIDR insertion and GnRH to CIDR removal and PGF from seven to five days and extending the period of proestrus from 60 to 72 h substantially increased timed-AI pregnancy rates in suckled beef cows.

Although the 5-d CO-Synch + CIDR approach was demonstrated to be a superior protocol for facilitating timed-AI in suckled beef cows than the 7-d protocol, the additional animal handling to deliver the second dose of PGF 12 hours after CIDR removal would limit its adoption by some beef producers. In suckled beef cows the necessity for two doses of PGF in the 5-d protocol was confirmed because when only a single administration of PGF (either cloprostenol sodium or dinoprost tromethamine) was given, timed-AI pregnancy success was reduced (1xPGF; 53.1%; 2xPGF; 69.0%).[2] Furthermore, it was demonstrated that a single administration of PGF was not effective at inducing luteolysis in all suckled beef cows.[3] Therefore, subsequent studies by various laboratories investigated how to modify the $PGF_{2\alpha}$ delivery in the 5-d protocol to improve "user-friendliness" of the protocol. Initial investigations on modification of $PGF_{2\alpha}$ delivery focused on determining the shortest interval between PGF administrations. Cruppe et al[4] compared timed-AI pregnancy rates in suckled beef cows (n = 254) with the 5-d protocol when two doses of PGF were administered at either a 12 or 2 h interval. In addition, the decline in progesterone concentrations following administration of PGF doses at a two or 12 h interval was evaluated. Timed-AI pregnancy rates were similar between the 2h and 12h PGF treatments (60.8% and 58%, respectively). Also, rate and incidence of luteal regression was similar between PGF intervals. These studies indicated that a reduction of the interval to the second PGF treatment from 12 to two hours in the 5-d CO-Synch + CIDR program did not influence timed-AI pregnancy rate or the occurrence of luteal regression. However, reduced AI pregnancy rates when the interval of PGF administration was less than six h apart was recently reported; however a treatment by location interaction was observed within this study.[5]

Although reducing the interval of PGF delivery to two h marginally improved the practical application of the 5-d protocol, it still required an additional animal handling that might impede producer adoption. Therefore, two experiments were conducted to determine if two 25 mg doses of PGF could be

delivered simultaneously (CoPG; 50 mg). The ability to simultaneously deliver two doses of $PGF_{2\alpha}$ without sacrificing timed-AI pregnancy rates would be more acceptable to producers and increase the adoption of the 5-d protocol. In the first experiment,[6] 662 postpartum beef cows at five locations were synchronized with the 5-d CO-Synch + CIDR protocol with the two doses of PGF given either at a eight h interval (8hPG), two h interval (2hPG), or both doses given coincident with CIDR removal (CoPG). Timed-AI pregnancy rates did not differ between treatments (8hPG, 66.1%; 2hPG, 65.5%; and CoPG; 69.7%). The second study was a large multistate project conducted in 2,465 postpartum beef cows at 13 herds in eight states.[7] Cows were enrolled in the 5-d CO-Synch + CIDR protocol and assigned to receive two 25 mg doses of PGF eight h apart (8hPG), two 25 mg doses of PGF delivered simultaneously (CoPG), or a single 25 mg dose of PGF (1xPG). Timed-AI pregnancy rates were greater ($P < 0.05$) for the 8hPG (55%) than the 1xPG (48%) with the CoPG (51%) being intermediate and not different from either treatment. Hence, 50 mg of PGF is required in the 5-d protocol, however pregnancy rates did not differ if 50 mg was administered simultaneously at CIDR removal (CoPG) or if doses were given at an eight h interval.

As outlined above, the 5-d CO-Synch + CIDR protocol is an effective method for facilitating timed-AI in postpartum beef cows. This approach to synchronization has been proven more effective than the 7-d approach and given the ability to deliver two doses of PGF simultaneously, is equally convenient in terms of cattle handling. In addition, the 5-d CO-Synch approach has been demonstrated to be an effective method for facilitating timed-AI and resynchronization in lactating dairy cows.[8,9,10] Table 2 summarizes timed-AI pregnancy rates achieved in postpartum beef cows with the 5-d CO-Synch + CIDR protocol and Figure 1 outlines our recommended approach when using the 5-d protocol in beef cows.

Application of the 5-d CO-Synch + CIDR protocol in heifers

Yearling heifers continue to be the most difficult class of beef cattle to work with relative to consistency and predictability of timed-AI. As in postpartum cows however, the 5-d CO-Synch + CIDR protocol has been proven as an effective and consistent approach to synchronize estrus and ovulation in beef and dairy heifers. In beef heifers, the 5-d protocol has been investigated as a Select Synch approach (estrous detection and AI), CO-Synch approach (strict timed-AI), or a Select Synch + Timed-AI (estrous detection and AI followed by timed-AI in heifers not observed in estrus). With each of these approaches, the 5-d protocol has been demonstrated to be as or more effective than other approaches to estrous synchronization currently available. Table 3 summarizes the various experiments that have compared AI pregnancy rates in beef heifers following synchronization with the 5-d approach or another method of synchronization. In summary, the 5-d protocol has been demonstrated to be more effective than the 7-d approach to estrous synchronization in beef heifers.[11,12] Furthermore, the 5-d approach is as effective as the longer, 14-d CIDR approaches to estrous synchronization and is of shorter duration.

Although the 5-d CIDR approach has been demonstrated as an effective method to synchronize estrous in heifers, efforts to optimize the 5-d approach have been and are currently being conducted. Among these include determining: 1) the necessity of GnRH administration at CIDR insertion, 2) the appropriate delivery of PGF at CIDR withdrawal, and 3) the ideal interval from CIDR removal to timed-AI. These investigations have been conducted in both beef and dairy heifers and are summarized in Table 4. One of the most critical experiments was that of Kasimanickam et al,[13] which indicated that timed-AI pregnancy rates in beef heifers were increased when the interval from CIDR removal to timed-AI was reduced from 72 h (as in cows) to 56 h. Although this requires additional investigations, based on these data and the collective summation of data generated using the 5-d approach, the ideal time for timed-insemination may lie between 56 and 66 h after CIDR removal. The necessity of GnRH at CIDR insertion and PGF requirements at CIDR withdrawal are still unclear as results of various investigations are mixed. Currently, we are conducting a large multi-state investigation to further clarify this question in beef heifers. Due to the variation in approaches taken with the 5-d protocol in heifers, recommending the "ideal" protocol is difficult. To maximize AI pregnancy rates, we recommend the 5-d Select Synch + CIDR & timed-AI protocol with two doses of PGF given at CIDR removal, estrous detection and AI

following CIDR removal, with timed-AI in heifers that fail to show estrus at 72 h (concurrent with GnRH administration). For strict timed-AI, some questions remain, and are being investigated, as to the best approach. For now, GnRH administration is still recommended at CIDR insertion. Likely, two simultaneous doses of PGF at CIDR removal will be effective in heifers, as it has been demonstrated in cows, albeit this PGF delivery has not directly investigated in beef heifers. The interval from CIDR removal to timed-AI is still debatable. Based on estrous expression data and previous studies, we believe that for strict timed-AI, insemination concurrent with GnRH administration at 60-66 h after CIDR removal would be ideal (Figure 2). This interval to timed-AI is currently being investigated.

Summary

The 5-d CO-Synch + CIDR protocol was developed based on sound physiological principles. Fundamental research indicated that pregnancy success would be improved if preovulatory estradiol concentrations were maximized and duration of follicular dominance minimized. Reducing the interval from GnRH and CIDR insertion to CIDR removal from seven to five days reduced the duration of follicular dominance and allowed the proestrus interval to be extended; thus maximizing preovulatory estradiol concentrations. Implementation of the 5-d protocol in beef and dairy cows and heifers has demonstrated that it is an effective method of estrous synchronization that yields greater AI pregnancy rates than most other synchronization approaches. In beef cows, that ability to deliver two doses of PGF simultaneously at CIDR removal simplifies the 5-d approach. Although the 5-d approach is still being optimized in heifers, in its current recommended form (Figure 2), it still delivers acceptable and consistent AI pregnancy rates.

Acknowledgements

Appreciation is expressed to Select Sires Inc., Zoetis (formerly Pfizer Animal Health), and IVX Animal Health for funding and donation of pharmaceuticals to support the various experiments presented within this review. In addition, the authors would like to thank the numerous individuals and other laboratories that assisted with research presented herein.

Figure 1. Illustration of the recommended 5-d CO-Synch + CIDR protocol in postpartum beef cows.

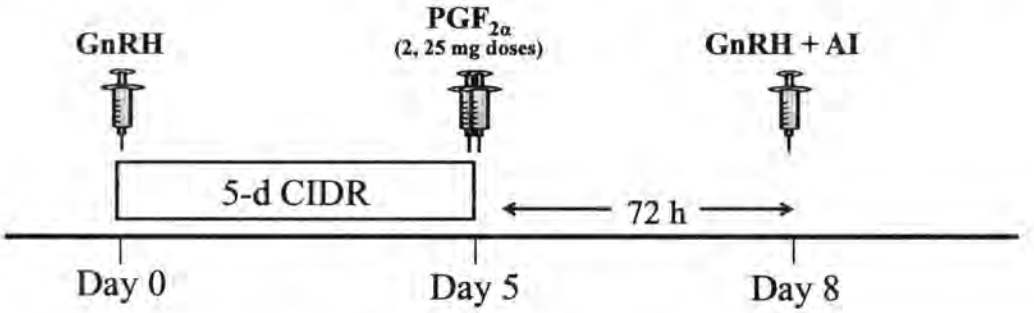


Figure 2. Illustration of the recommended 5-d CO-Synch + CIDR protocol in beef heifers.

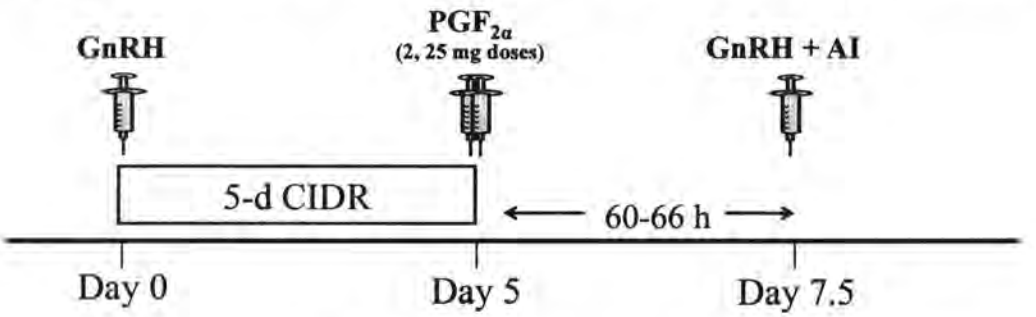


Table 1. Reproductive performance of postpartum beef cows synchronized with either the 7-d or 5-d CO-Synch + CIDR* program (Adapted from Bridges et al., 2008)

Variable	7-d AI @ 60 vs. 5-d AI @ 72 2005		7-d AI @ 60 vs. 5-d AI @ 72 2006	
	7-d	5-d	7-d	5-d
N	111	105	201	199
Cyclic, %	11.7	8.6	63.2	65.8
Timed AI pregnancy rates, %	66.7[a]	80.0[b]	56.2[a]	65.3[b]

* Cows in all experiments received 100 mg of GnRH and a CIDR on either Day-7 (7CO) or Day-5 (5CO). On Day 0 the CIDR was withdrawn, and PGF was given. A second dose of PGF was given to all cows approximately 12 h later.
[a,b] Means differ between treatments within experiment, $P < 0.05$.

Table 2. Summary of data in postpartum cows using the 5- or 7-d CO-Synch + CIDR protocol with various luteolytic treatments.

Synchronization protocol[a] Luteolytic treatment[b]	5-d-72 2xPGF	7-d-60 2xPGF	5-d-72 CoPG	5-d-72 1xPGF	5-d-72 1xCLP
Bridges et al., 2008[1] (n = 216)	80.0	66.7	-	-	-
Bridges et al., 2008[1] (n = 400)	65.3	56.2	-	-	-
Cruppe et al., 2010a[4] (n = 254)	59.4	-	-	-	-
Culp et al., 2009[14] (n = 194)	74.2	-	-	-	-
Souto et al., 2009[3] (n = 254)	69.0	-	-	-	-
Kasimanickam et. al., 2009[2] (n = 830)	69.0	-	-	52.0	54.3
Cruppe et al., 2010b[6] (n = 662)	65.8	-	69.2	-	-
Peel et al., 2010a[15] (n = 419)	57.5	-	-	-	-
Peel et al., 2010b[16] (n = 642)	50.7	-	-	-	-
Bridges et al., 2012[7] (n = 2,465)	55	-	51	48	-
Peel et al., 2012[5] (n = 894)	53.9	-	-	-	-
Dias et al., 2012 unpublished (n = 123)	-	-	67.5	-	-
Total (n = 7,353)	**63.6**	-	**62.6**	-	-

[a] Number after the final hyphen (e.g. 5-d-72) indicates time of GnRH treatment and timed-AI after removal of CIDR insert.

[b] 2xPGF = PGF (25 mg) or cloprostenol sodium (500 μg) given at CIDR insert removal and a second dose between 2 and 12 h later; CoPG = two doses of PGF given concurrently at CIDR insert removal; 1xPGF = one dose of PGF given at CIDR insert removal; 1xCLP = one dose of cloprostenol sodium given at CIDR insert removal.

Table 3. Comparison of heifer AI pregnancy rates when estrous was synchronized with the 5-d approach or another protocol.

Experiment	Comparison	n	AI PR, %	*P*-value
Helser et al., 2006[17]	7-d Select Synch + CIDR *(1xPGF[1])*	79	58.2	> 0.10
	5-d Select Synch + CIDR *(1xPGF)*	80	61.3	
Wilson et al., 2007[11]	7-d CO-Synch + CIDR *(1xPGF)*	109	47.0	= 0.02
	5-d CO-Synch + CIDR *(1xPGF)*	108	62.0	
Ahmadzadeh et al., 2010[18]	7-d CO-Synch + CIDR *(1xPGF)*	147	52.0	= 0.09
	5-d CO-Synch + CIDR *(1xPGF)*	145	62.5	
Bridges & Lake, 2011[19]	14-d CIDR Select & TAI *(1xPGF)*	153	70.4	> 0.10
	5-d Select Synch + CIDR & TAI *(2xPGF[2])*	150	70.4	
Sparks et al., 2012[12]	7-d Select Synch + CIDR & TAI *(2xPGF)*	297	43.7[a]	< 0.05
	7-d Mod. Select Synch + CIDR & TAI *(2xPGF)*	374	58.4[b]	
	5-d Select Synch + CIDR & TAI *(2xPGF)*	368	57.1[b]	
Perry et al., 2012[20]	14-d CIDR-PG *(1xPGF)*	257	53.3	= 0.13
	5-d CO-Synch + CIDR *(2xPGF)*	267	62.5	
	PG 6-d CIDR *(1xPGF)*	257	56.9	

[1] *1xPGF* = single 25 mg dose of PGF at CIDR removal
[2] *2xPGF* = two 25 mg doses of PGF given 0 to 12 h apart at CIDR removal

Table 4. AI pregnancy rates with varying approaches to the 5-d CO-Synch + CIDR protocol in beef and dairy heifers.

Experiment	Comparison	n	AI PR, %	*P*-value
Rabaglino et al., 2010a[21] *(dairy)*	5-d CO-Synch + CIDR *(1xPGF)*[1]	295	46.1	> 0.10
	5-d CO-Synch + CIDR *(2xPGF)*[2]	298	48.6	
Rabaglino et al., 2010a[21] *(dairy)*	5-d CO-Synch + CIDR Resynch *(1xPGF)*	54	51.8	= 0.07
	5-d CO-Synch Resynch *(1xPGF)*	56	39.3	
Rabaglino et al., 2010a[21] *(dairy)*	5-d CO-Synch + CIDR *(1xPGF)*	416	60.3	-
Rabaglino et al., 2010b[22] *(dairy)*	5-d CO-Synch + CIDR *(1xPGF)*	165	59.4	> 0.10
	5-d CO-Synch + CIDR + Flunixin *(1xPGF)*	158	59.5	
Lima et al., 2011[23], Expt 1 *(dairy)*	5-d CO-Synch + CIDR *(GnRH + 1xPGF)*[3]	298	52.5	= 0.83
	5-d CO-Synch + CIDR *(NoGnRH + 1xPGF)*[4]	307	54.1	
Lima et al., 2011[23], Expt 2 *(dairy)*	5-d CO-Synch + CIDR *(GnRH + 1xPGF)*	651	58.4	= 0.08
	5-d OV-Synch + CIDR *(NoGnRH + 1xPGF)*	644	55.4	
Peterson et al, 2011[24] *(beef)*	5-d CO-Synch + CIDR *(1xPGF)*	264	54.2	= 0.06*
	5-d CO-Synch + CIDR *(2xPGF)*	298	62.1	
Kasimanickam et al., 2012[13] *(beef)*	5-d CO-Synch + CIDR-72 h[5] *(2xPGF)*	544	55.9	< 0.01
	5-d CO-Synch + CIDR-56 h[6] *(2xPGF)*	554	66.2	
Abreu et al., 2012[25] & Cruppe et al., 2012 (unpub.) *(beef)*	5-d CO-Synch + CIDR *(NoGnRH + 1xPGF)*	416	54.7	= 0.25
	5-d CO-Synch + CIDR *(GnRH + 1xPGF)*	413	50.7	
Lima et al., 2012[26] *(dairy)*	5-d CO-Synch + CIDR *(NoGnRH +1xPGF)*	711	52.9[a]	= 0.01
	5-d CO-Synch + CIDR *(NoGnRH +2xPGF)*	696	55.0[a]	
	5-d CO-Synch + CIDR *(2xPGF)*	711	61.7[b]	
Kasimanickam et al., 2012 (unpub.) *(beef)*	5-d CO-Synch + CIDR *(NoGnRH +1xPGF)*	272	51.8	NA
	5-d CO-Synch + CIDR *(NoGnRH +2xPGF)*	223	52.2	
	5-d CO-Synch + CIDR *(GnRH +1xPGF)*	248	62.8	
	5-d CO-Synch + CIDR *(GnRH +2xPGF)*	224	58.4	
Kasimanickam et al., 2012 (unpub.) *(dairy)*	5-d CO-Synch + CIDR *(NoGnRH +1xPGF)*	280+	51.2	NA
	5-d CO-Synch + CIDR *(NoGnRH +2xPGF)*	280+	51.9	
	5-d CO-Synch + CIDR *(GnRH +1xPGF)*	280+	53.9	
	5-d CO-Synch + CIDR *(GnRH +2xPGF)*	280+	54.5	

[1] *1xPGF* = single 25 mg dose of PGF at CIDR removal
[2] *2xPGF* = two 25 mg doses of PGF given 0 to 12 h apart at CIDR removal
[3] *GnRH* = administration of GnRH at CIDR insertion
[4] *NoGnRH* = no administration of GnRH at CIDR insertion
[5] 72 h = timed-AI conducted 72 h after CIDR removal
[6] 56 h = timed-AI conducted 56 h after CIDR removal
* Significant treatment x location interaction (P < 0.01)

References

1. Bridges GA, Helser LA, Grum DE, et al: Decreasing the interval between GnRH and PGF2alpha from 7 to 5 days and lengthening proestrus increases timed-AI pregnancy rates in beef cows Theriogenology 2008;69:843-851.
2. Kasimanickam R, Day ML, Rudolph JS, et al: Two doses of prostaglandin improve pregnancy rates to timed-AI in a 5-day progesterone-based synchronization protocol in beef cows. Theriogenology 2009;71:762-767.
3. Souto LA, Maquivar M, Mussard ML, et al: Fertility and luteal regression with 5-d CIDR synchronization programs in postpartum beef cows using differing luteolytic treatments [abstract]. J Anim Sci 2009;87(Suppl 2):372.
4. Cruppe LH, Maquivar M, Jinks EM,et al: The influence of two doses of $PGF_{2\alpha}$ given at 2 or 12 hour intervals on luteolysis and pregnancy rate to timed AI with the 5-d CO-Synch + CIDR program [abstract]. J Anim Sci 2010a;88(E-Suppl 2):767.

5. Peel, RK, Seabrook JL, Seidel GE Jr., et al: Effect of 2, 4,and 5-hour interval between 2 prostaglandin $F_{2\alpha}$ injections administered with the 5-d CO-Synch + CIDR protocol on pregnancy rates in beef cows. Prof Anim Sci 2012;28:623-627.
6. Cruppe LH, Souto LA, Maquivar M, et al: Use of two coincident doses of $PGF_{2\alpha}$ with the 5-d CO-Synch + CIDR estrous synchronization program [abstract]. J Anim Sci 2010b;88(E-Suppl 2):768.
7. Bridges GA, Ahola JK, Brauner C, et al: Determination of the appropriate delivery of PGF2α in the 5-day CO-Synch + CIDR protocol in suckled beef cows. J Anim Sci 2012;90:4814-4822.
8. Santos JE, Narciso CD, Rivera F, et al: Effect of reducing the period of follicle dominance in a timed artificial insemination protocol on reproduction of dairy cows. J Dairy Sci 2010;93:2976-2988.
9. Bisinotto RS, Ribeiro ES, Martins LT, et al: Effect of interval between induction of ovulation and artificial insemination (AI) and supplemental progesterone for resynchronization on fertility of dairy cows subjected to a 5-d timed AI program. J Dairy Sci 2010;93:5798-5808.
10. Ribeiro ES, Monteiro AP, Lima FS, et al: Effects of presynchronization and length of proestrus on fertility of grazing dairy cows subjected to a 5-day timed artificial insemination protocol. J Dairy Sci 2012;95:2513-2522.
11. Wilson KN, Day ML, Whittier WD, et al: Comparison of 5-day or 7-day CIDR-based estrous synchronization systems for fixed-time AI in beef heifers [abstract]. J Anim Sci 2007;85(Suppl 2):43.
12. Sparks BL, Lake SL, Gunn PJ, et al: Effects of prostaglandin $F_{2\alpha}$ administration at CIDR insertion on artificial insemination pregnancy rates in beef heifers. Prof Anim Sci 2012;28:552-559.
13. Kasimanickam R, Asay M, Firth P, et al: Artificial insemination at 56 h after intravaginal progesterone device removal improved AI pregnancy rate in beef heifers synchronized with five-day CO-Synch + controlled internal drug release (CIDR) protocol. Theriogenology 2012;77:1624-1631.
14. Culp KC, Lemenager RP, Claeys MC,et al: Efficacy of the 5 day CO-Synch estrous synchronization protocol with or without the inclusion of a CIDR in beef cows [abstract]. J Anim Sci 2009;87(E-Suppl 2):466.
15. Peel, RK, Ahola JK, Whittier WD, et al: Effect of calf separation during the 12-hour interval between 2 $PGF_{2\alpha}$ injections when using the 5-d CO-Synch + CIDR protocol on pregnancy in beef cows. Prof Anim Sci 2010a;26:534-539.
16. Peel, JK, Whittier, JC, Enns,et al: Effect of 6- versus 12-hour interval between two prostaglandin $F_{2\alpha}$ injections administered with the 5-d CO-Synch + controlled internal drug-release protocol on pregnancy in beef cows. Prof Anim Sci 2010b;26:307-312.
17. Helser LA, Bridges GA, Grum DE, et al: Effect of decreasing the interval from GnRH to PGF2a and lengthening proestrus on reproductive performance in GnRH-CIDR-PGF2a synchronization programs [abstract]. J Anim Sci 2006;84(Suppl 1):432.
18. Ahmadzadeh A, Gunn D, Hall DB, et al: Evaluation of 5-day versus 7-day CIDR treatment on reproductive outcomes of beef heifers using a modified timed-AI protocol [abstract]. J Anim Sci 2010;88(E-Suppl)2:393.
19. Bridges GA, Lake SL: Comparison of the CIDR Select and 5-day Select Synch + CIDR protocols that included limited estrus detection and timed insemination for synchronizing estrus in beef heifers. Prof Anim Sci 2011;27:141-146.
20. Perry GA, Grant JK, Walker JA, et al: Comparison of three CIDR-based fixed-time AI protocols for beef heifers [abstract]. J Anim Sci 2012;90(Suppl 3):237.
21. Rabaglino MB, Risco CA, Thatcher MJ, et al: Application of one injection of prostaglandin F(2alpha) in the five-day Co-Synch+CIDR protocol for estrous synchronization and resynchronization of dairy heifers. J Dairy Sci 2010a;93:1050-1058.
22. Rabaglino MB, Risco CA, Thatcher MJ, et al: Use of a five-day progesterone-based timed AI protocol to determine if flunixin meglumine improves pregnancy per timed AI in dairy heifers. Theriogenology 2010b;73:1311-1318.
23. Lima FS, Ayres H, Favoreto MG, et al: Effects of gonadotropin-releasing hormone at initiation of the 5-d timed artificial insemination (AI) program and timing of induction of ovulation relative to AI on ovarian dynamics and fertility in dairy heifers. J Dairy Sci 2011;94:4997-5004.
24. Peterson C, Alkar A, Smith S, et al: Effects of one versus two doses of prostaglandin F2alpha on AI pregnancy rates in a 5-day, progesterone-based, CO-Synch protocol in crossbred beef heifers. Theriogenology 2011;75:1536-1542.
25. Abreu FM, Cruppe LH, Biehl MV, et al: The requirement of GnRH at the onset of the 5-d Select Synch + CIDR program in beef heifers [abstract]. J Anim Sci 2012;90(Suppl 3):576.
26. Lima FS, Ribeiro ES, Bisinotto RS, et al: Effects of GnRH and administering number of PGF2α doses in the 5-d timed AI program on ovarian responses and fertility of dairy heifers [abstract]. J Dairy Sci 2012;95(Suppl 2):576.

Cost and value beef heifers bred using fixed time artificial insemination or short season natural service

David E. Cupps
Barry County Veterinary Service, PC, Cassville, MO

Artificial insemination (AI) of beef heifers is considered a 'best practice' for several reasons, including more consistent birth weights, better genetics and producers not having to own a "heifer bull". The adoption of AI technology is far from universal, with one recent author stating that fewer than five percent of beef cattle were bred by AI.[1] Recent advances in estrus synchronization techniques have made appointment breeding with a fixed time AI (FTAI) a more useful tool to incorporate AI into a producer's production system.

In this paper the author will try to determine the cost and the value of heifers bred using FTAI versus heifers bred using a short season (45 day) natural service period (45NS).

What is cost of a heifer bred with FTAI versus a heifer bred in a 45 day natural service period?

At our practice, when talking with clients, we found two primary reasons for not using AI, those being perceived higher cost and increased labor (both skill and time). In an effort to encourage our clients to utilize AI, our practice has developed and is providing a "turnkey" service for FTAI in beef heifers. In an effort to address the concern of cost, we developed a partial budget spreadsheet to compare a fixed time AI (FTAI) protocol with using a bull in a short season natural service system.

Setting up the spreadsheet we tried to consider all of the costs involved in both systems. The model considers variable inputs classified into three categories, temporal, production and financial. The primary driver of the model is the number of pregnant females a producer wants for replacements in his herd. From this number, we calculate back using assumed pregnancy rates of the two systems to determine how many heifers need to be saved or purchased for the replacement program. The heifers that do not conceive are marketed as feeder heifers. In the model heifers are basically backgrounded and "Oh, by the way" we'll do a one-time FTAI and keep the pregnant females. The comparison is a group of heifers that are managed the same way but bred to a bull using a short season (45 day) breeding period. Diagnosis of pregnancy 60 days after the last possible breeding date is the end point of the cost analysis.

The temporal variables in the model are:

1. Date of selection or purchase for the breeding program. This establishes a beginning date to allow calculation of total costs daily feed and maintenance and total animal gain in the development period.
2. Date of FTAI or bull turn in. This establishes a date for when breeding begins.
3. Date of pregnancy determination and culling. This establishes the end point of the costs associated with raising the heifers. It also is used to calculate the expected weight of the cull heifers and the total feed and maintenance costs.

The financial variables are:

1. Number of bred females required to meet herd replacement or expansion. This number along with expected pregnancy percentages is used to calculate how many females are selected to enter the model.
2. Price per hundredweight of the heifers at the time of selection or weaning. This allows the model to calculate the value of the heifers entered into the model.
3. Price slide per hundredweight for growing heifers. As the heifers grow the price per hundredweight usually goes down. This slide number allows the model to price the heifers at the culling after pregnancy determination.
4. Daily feed and maintenance costs per head per day. This number is utilized to get the expense of feeding and growing the heifers.
5. Cost of natural service sires. The cost of purchasing a bull is entered into the model here.

6. Annual feed and maintenance cost of natural service sires. The annualized cost of feeding and maintaining a bull is entered here.
7. Salvage value of bulls. Bulls purchased have a value on the cull bull market. This value is used to offset some of the initial cost of the bull unless a mortality event occurs.
8. Synchronization and AI expense per head. The cost of the AI protocol and semen cost is entered in the model here.
9. Value of any excess pregnant heifers. In the model AI pregnancy rates are usually set at an easily achieved level. If the AI success rate is in excess of the expected result, those heifers can usually be sold at this price which is generally much higher than the feeder cattle market culls would sold on. This number is not necessary for the model to work.

The production variables in the model are;

1. Weight of heifers selected or purchased for heifers development.
2. Expected 45 day natural service (45NS) pregnancy rate. This input is used to calculate how many non-breeding culls from the 45NS group will be available to sell.
3. Expected FTAI pregnancy rate. This input is used to calculate how many non-breeding culls in the FTAI group will be available to sell.
4. Average daily gain. Average daily gain allows the model to calculate the weight and subsequently the value of cull heifers at the time of culling.
5. Average useful life of natural service sires in years. In the model, the cost of natural service sires is amortized over the useful life. Annualized costs of natural service sires are applied to the cost of the 45NS heifers.
6. Involuntary cull rate of bulls. Bulls get injured or lose the ability to be effective breeders. This input enables the model to take that cost of early culling into account.
7. Heifer to bull ratio. Producers have a maximum number of heifers that they expect one bull to breed in a limited breeding period. Calculating the cost of 45NS requires this input.
8. FTAI pregnancy percentage in excess of the target percentage. This number is not necessary for the model, but it allows one to see how the cost of replacement females change when conception rates for AI bred heifers is better than target FTAI conception rates entered in the model.

Twenty five producers in our practice area were surveyed in the fall of 2011 for variable inputs for the following:

1. Cost of natural service sires
2. Annual feed and maintenance cost of bulls
3. Expected 45 day natural service pregnancy rate.
4. Daily feed and maintenance cost of heifers being developed
5. Involuntary cull rate of natural service sires
6. Useful life of natural service sires
7. Salvage value of natural service sires
8. Heifer to bull ratio in a 45NS system

All other variables were fixed at the following:

1. Number of heifers needed was fixed at 30 head.
2. Weight of heifers selected for replacement heifers development was set at 500 lbs.
3. November 1 was the beginning date for the growing period.
4. May 1 was the FTAI date and the date of bull turn in.
5. Average daily gain was fixed at 1.4 lbs. per head per day.
6. The price of heifers retained or purchased to develop was $120 per cwt.
7. Price slide of growing heifers was set at $8 per cwt.
8. Synchronization and AI expense was set at $60 per head inseminated.
9. Expected FTAI pregnancy rate was set at 50%.

10. Pregnancy confirmation/culling dates were set at 60 days after FTAI and 60 days after bull removal. Fixed time AI date of May 1 made the pregnancy confirm/cull date June 30. The 45NS bull turn in on May 1 made the pregnancy/cull confirm date Aug 14 for the 45NS heifers (45 day breeding season plus 60 days after bull removal).

The following are two screenshots of one replicate of the partial budget analysis. The first screenshot is the assumptions input section and results of those assumptions.

Barry County Veterinary Service, PC

David E. Cupps, DVM Voyd C. Brown, DVM M. Elizabeth Caldwell, DVM

Fixed Time Synchronized AI vs. 45 day Natural Service Breeding of Beef Heifers

Italic numbers in yellow cells are variable inputs and will change all the output values

Values in blue cells are result values that change with input changes.

Assumptions:

Number of bred heifers desired	*30*
Average wt of heifers (beginning of pre-breeding period)	*500*
Heifer feeder calf price	*$1.60*
Feed and Maintenance cost/head/day post weaning	*$1.40*
Average daily gain	*1.40*
Price slide per cwt.	*$8.00*
Natural service pregnancy rate (45 day)	*75%*
AI pregnancy rate (FTAI)	*50%*
Cost of bulls	*$3,500*
Useful life of bull years	*3*
Salvage value of bulls	*$1,200*
Involuntary cull rate of bulls %/yr	*20%*
Synchronization & AI Expense/hd	*$60.00*
Annual bull feed & maintenance cost/hd	*$650*
Heifer to bull ratio (45NS)	*20*

	Breeding season	
Weaning date	1st day	Preg check date
11/01/11	***05/01/12***	06/30/12
11/01/11	***05/01/12***	08/14/12

Results:

Days post weaning to preg check/cull date (FTAI)	242
Days post weaning to preg check/cull date (45NS)	287
Number of heifers needed for FTAI	60
Number of heifers needed for 45NS	40
Post weaning cost for FTAI system heifers	$338.80
Post weaning cost for 45NS system heifers	$401.80
Cull heifer price 60 days post breeding (FTAI)	$1.33
Cull heifer weight 60 days post breeding period (FTAI)	839
Cull heifer price 60 days post breeding period (45NS)	$1.28
Cull heifer weight 60 days post breeding period (45NS)	902
Number of bulls required for 45NS	2
Annual cost of bulls needed for 45NS	$2,220.00
Annual cost of Synchronizatin & AI	$3,600.00
Value of heifers kept for breeding (FTAI)	$48,000.00
Value of heifers kept for breeding (45NS)	$32,000.00
Cull heifer proceeds (FTAI)	$33,441.95
Cull heifer proceeds (45NS)	$11,530.05
Heifer feed/maintenance cost (FTAI)	$20,328.00
Heifer feed/maintenance cost (45NS)	$16,072.00

The second is a screenshot of the enterprise results:

Barry County Veterinary Service, PC

David E. Cupps, DVM Voyd C. Brown, DVM M. Elizabeth Caldwell, DVM

Snchronized AI heifer program vs All bull heifer program

Italic numbers in yellow cells are variable and will change all the output values

Enterprise results

	FTAI	45NS	FTAI + *10%* better conception rate
Expenses			
Value of retained heifers	$48,000.00	$32,000.00	$48,000.00
Feed & maintainence	$20,328.00	$16,072.00	$20,328.00
Bull expenses	0	$2,220.00	0
Synchronization & AI expense	$3,600.00	0	$3,600.00
Total Expense	$71,928.00	$50,292.00	$71,928.00
Income			
Sale of cull heifers	$33,441.95	$11,530.05	$26,753.56
Sale of excess bred heifers			$10,500.00
Net cost of bred replacements	$38,486.05	$38,761.95	$34,674.44
Net cost per head	$1,282.87	$1,292.06	$1,155.81

Market value of AI breed heifers at preg check ***$1,750.00***

# of excess AI bred heifers 6

In the survey, value of excess FTAI bred heifers was ignored.

Producers' input variables from this survey were used to run the spreadsheet model 25 repetitions. Average cost of producing a pregnant heifer using FTAI in this survey was $1445.87 versus $1449.72 to produce a pregnant heifer using natural service with a 45 day breeding window, a mean advantage of $3.85 for FTAI. The range of results was $174.22 disadvantage to $311.90 advantage for FTAI. These results were reported in an abstract at the World Buiatrics Congress in Lisbon, Portugal in June 2012[2].

Using this partial budget comparison spreadsheet model indicates that the cost of a pregnant heifer produced by using FTAI is not quantitatively different than using natural service for 45 days.

What is value of a heifer bred with FTAI versus a heifer bred in a 45 day natural service period?

Expected advantages would be from the following:

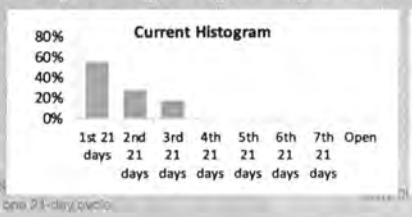
1. Earlier average calving dates result in older and subsequently heavier calves at weaning. If all cows are bred the same day versus being bred over a 45 day period, the resulting calving dates of the FTAI-sired pregnancies will be earlier. This earlier calving will result in older, heavier calves at weaning and marketing.
2. Earlier calving dates result in longer postpartum intervals prior to beginning of breeding season for the second calf, improving second and subsequent conception rates (reproductive momentum).[6] Heifers calving from FTAI will, on average, have a longer postpartum interval. This longer time allows the heifers to prepare for the following breeding and "reproductive momentum" is established in this group where early calving can be maintained in subsequent production cycles.
3. Genetic advantage of high accuracy sires. Expected progeny differences (EPDs) for proven AI sires are much more reliable with accuracies up to .90. One would expect that those bred by AI will produce calves more likely to grow at faster rates producing heavier calves at weaning and marketing.
4. Expected improved dystocia rates. We can expect high accuracy AI sires to produce more consistent low birth weights. The result would be fewer dystocias and improved survival rates and increased second calf pregnancy rates.

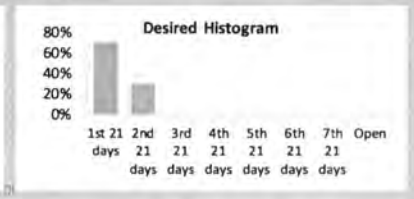
Earlier average calving dates result in older and subsequently heavier calves at weaning.

Partial budget spreadsheets can also be used to calculate the financial advantages of heifers bred by FTAI and subsequently calving in an extremely short season. Dr. Bob Larson and his colleagues at the Kansas State University Beef Cattle Institute have published a model that evaluates calving histograms.[3]

KSU College of Veterinary Medicine - Production Medicine
Value of Improving Pregnancy Distribution

Herd size (# cows exposed to bulls)	30
Start of calving (mm/dd)	3/1
Weaning date (mm/dd)	11/1
ADG (birth to weaning)	2.2
Price slide ($/cwt)	$10.00
Price ($/cwt) - 500 lb. steers	$150.00





It is unlikely that more than 70% become pregnant in one 21-day cycle.

% Pregnant	Current	% of open cows becoming pregnant	Weight at Weaning	Total of Value of Calves	Desired	% of open cows becoming pregnant	Total of Value of Calves
1st 21 days	55%	55%	592	$13,675	70%	70%	$17,405
2nd 21 days	28%	62%	546	$6,877	30%	100%	$7,368
3rd 21 days	17%	100%	500	$3,822	0%	#DIV/0!	$0
4th 21 days	0%	0%	453	$0	0%	#DIV/0!	$0
5th 21 days	0%	0%	407	$0	0%	#DIV/0!	$0
6th 21 days	0%	0%	361	$0	0%	#DIV/0!	$0
7th 21 days	0%	0%	315	$0	0%	#DIV/0!	$0
Open	0%				0%		

Current scenario (avg. weaning wt.)	563
Desired scenario (avg. weaning wt.)	578
Current scenario ($ value of weaned calves)	$24,374
Desired scenario ($ value of weaned calves)	$24,773
Value of moving to desired calving distribution (ranch)	$399
Value of moving to desired calving distribution (per calf)	$13.30

Their model compares calf weaning weights and value for calves born during one of seven 21-day intervals. The model shows that moving more cows into the earlier 21-day periods of the calving season make the herd more profitable. The example above uses a more typical 65 day calving period versus a 45NS season.

In their model, average calving dates are calculated to be the mid-point of each 21-day estrous cycle or 10.5 days after the beginning of each 21-day period. In the above example a group with a typical calving distribution is compared with a group where the breeding was optimal using natural service. In the desired column we would have a goal of 70% in the first 21-day period and the remaining 30% in the second 21-day period. This example shows how improving the calving distribution will increase return to the producer.

The model Larson et al use assumes the heifers conceive over a 21 day period. They then assign an expected average calving date of 283 days (average gestation period) plus 10.5 days after the beginning of that 21 day period. In a FTAI system all heifers are bred on the same day so one would have to assume the average calving date in that model would be 283 days after the date of FTAI. Even if a producer used a 21 day natural service or 21 day observed estrus AI system, FTAI bred heifers would have a 10.5 day advantage over the heifers who conceive in a 21 day breeding period. A report from the

Nobel Foundation in 2010 showed FTAI heifers calving over a 28 day period. The authors point out that the weighted average gestational length for this group of heifers was 279 days or four days fewer than the traditional 283 day average. Using this data the advantage for FTAI would appear to be even greater.[4]

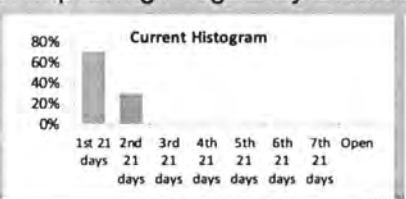
Producers rarely rely on a 21 day breeding period. The more normal practice is 60-90 day seasons. The average age advantage is exacerbated when longer natural service periods are used. The 45 day natural service period used in our cost models will improve the average calving date over traditional 60-90 day breeding periods but still have a marked disadvantage compared to FTAI.

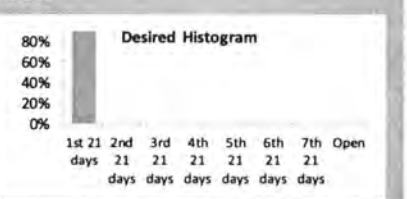
In the following spreadsheet/histogram model, we modify Larson's model to compare a 45 day natural service to a FTAI system. We have modified the formula for average calving date by assuming the average calving date for the FTAI group is day 1. Using a 45 day natural service breeding system in the model, we assume 70% of the pregnancies are from first service (first 21 days of the breeding period) and 30% from second service (last 24 days of breeding period). The average calving dates for 70% of the calves will be 10.5 days from the start of the calving season and 30% of the heifers will, on average, calve 31.5 (10.5+21) days from the start of the calving season. In this model the average calving date for a group of 45NS bred heifers is day 17 (.70 X 10.5) + (.30 X 31.5). This would give the FTAI calves a 17 day advantage for age at weaning. What is the economic value of those 17 days? Larson's model assumes 2.2 lbs. per day of age, the extra 17 days of age translates into 34 lbs. heavier calves at weaning assuming the calves from either system will gain at the same rate regardless of sire. If one uses $1.00 per pound for additional weight, the FTAI bred heifers have an advantage of $33.83 per head.

KSU College of Veterinary Medicine - Production Medicine

Value of Improving Pregnancy Distribution

Herd size (# cows exposed to bulls)	30
Start of calving (mm/dd)	3/1
Weaning date (mm/dd)	11/1
ADG (birth to weaning)	2.2
Price slide ($/cwt)	$10.00
Price ($/cwt) - 500 lb. steers	$150.00





% Pregnant	Current	% of open cows becoming pregnant	Weight at Weaning	Total of Value of Calves	Desired	% of open cows becoming pregnant	Total of Value of Calves
1st 21 days	70%	70%	592	$17,405	100%	100%	$25,788
2nd 21 days	30%	100%	546	$7,368	0%	#DIV/0!	$0
3rd 21 days	0%	#DIV/0!	500	$0	0%	#DIV/0!	$0
4th 21 days	0%	#DIV/0!	453	$0	0%	#DIV/0!	$0
5th 21 days	0%	#DIV/0!	407	$0	0%	#DIV/0!	$0
6th 21 days	0%	#DIV/0!	361	$0	0%	#DIV/0!	$0
7th 21 days	0%	#DIV/0!	315	$0	0%	#DIV/0!	$0
Open	0%				0%		

Current scenario (avg. weaning wt.)	578
Desired scenario (avg. weaning wt.)	614
Current scenario ($ value of weaned calves)	$24,773
Desired scenario ($ value of weaned calves)	$25,788
Value of moving to desired calving distribution (ranch)	$1,015
Value of moving to desired calving distribution (per calf)	$33.83

Earlier calving dates result in longer postpartum intervals prior to beginning of breeding season for the second calf, improving second calf and subsequent conception rates (reproductive momentum).[5]

Pregnancy rates of second calf heifers are directly related to postpartum interval. Various authors emphasize calving first-calf heifers earlier than the adult cow herd to improve the number of heifers that begin cycling prior to the start of the following breeding season.[6] The 17 day advantage for the FTAI heifer group also gives that group a 17 day advantage in postpartum interval. If one takes the 365 days of one year and subtracts a 283 day average gestation, a postpartum cow has 82 days to breed back to maintain a 365 day calving interval. Using FTAI one should get approximately the same effect of starting the primaparous heifer breeding season 17 days earlier. If the producer elects to calve his heifers three weeks earlier than the cow herd, FTAI will exacerbate the early calving effect on first calf heifer cyclicity. The extra 17 days postpartum the FTAI group has should improve second calf pregnancy rates. Pregnancy rates increase as postpartum intervals increase.[9] Heifers that calve in the last part of the calving period have a shorter postpartum period to prepare their reproductive tract for the following breeding season.

We will assume the distribution of natural service pregnancies are 70% in the first 21 day period and the remaining 30% in the following 24 day period. By using the average calving dates of +10 days for 70% of the heifers and +35.5 for the 30% in the second 24 day period, 70% of the heifers are 73 days postpartum when the following breeding season begins and 30% of the heifers are (on average) only 52 days postpartum when the following breeding season begins. Using these assumptions second calf pregnancy rates of the 45NS heifers calving in the second 24 day period will be impaired due to the shorter calving to breeding interval. We will assume the 70% of the heifers calving in the first 21 day period will have a 90% rebreed rate while the 30% of the 45NS heifers calving in the second 24 day period will have a rebreed rate of 60%. The 45NS heifers would then have an overall pregnancy rate of 81% ([90%X70%] + [60%X30%] = 81%). Using the same assumptions, the FTAI group would have a second calf pregnancy rate of 90%.

In the cost model we use for producing pregnant heifers, the cost of those heifers averaged about $1450 at pregnancy diagnosis. If that $1450 heifer is culled after one calf, her cost is amortized over one calf, while a heifer that produces a calf every year for the next five years can have her initial cost amortized against five calves or $290 per year ($1450/5=$290). The penalty for having to cull a non-pregnant animal after one calf is $1160 ($1450-$290).

In the FTAI group the annualized cost of rearing is $406. This is calculated by the following; 90% of the heifers breed back and assume an annualized cost of $290 and 10% do not rebreed and are culled and assume an annualized cost of rearing of $1450. The FTAI group average cost of rearing for that year is ($290 X 90%) + ($1450 X 10%)=$406. Making the same assumption for the 45NS group the average annualized cost of rearing is ($290 X 81%) + ($1450 X 19%) = $510. The advantage of FTAI is ($510 - $406) or $104 per head.

Genetic advantage of high accuracy AI sires

When one compares available AI sires with purebred unproven bulls, the primary difference is accuracy of the EPD numbers. Using data provided by the University of Missouri extension service from their Southwest Missouri Tested Bull Sale from the last five years,[7] we used data from a subset of bulls that producers consider to be "calving ease bulls". (For the Missouri Sho-Me Select heifer program Calving Ease Direct for Angus sires must be 7 or above to be used.) We extracted the data for the bulls sold in the sales in the last five years and used only bulls with Calving Ease Direct 7 or greater. Estimated progeny difference data for Calving Ease Direct and EPD weaning from these sale bulls was compared with a commercial bull stud's "calving ease" bull list.[8]

Following is the comparison:

Comparison of Commercial AI stud Angus "calving ease sires" with natural service bulls sold at the SW Missouri Performance Tested Bulls Sales (5 yrs data)				
		CE direct	WW (lbs)	$W
Commercial AI stud	*	+11	+53	$38.45
High accuracy	(*accuracy*)	0.79	0.87	
Commercial AI stud	*	+11	+57	$37.31
Low accuracy	(*accuracy*)	0.42	0.47	
SW MO Tested bulls	**	+8.5	+52.2	$29.24
	(*accuracy*)	0.227	0.226	

* ABS Global January 2013 sire catalog

** Eldon Cole, University of Missouri area livestock extension agent

Observing the data, we cannot conclude that the AI sires have any advantage for siring calves that will have heavier weaning weights. Obviously if one uses one of the high accuracy AI sires, the predicted results are much more likely to happen. Using the low accuracy bulls the calving ease and weaning weight result attained is much less predictable. The correlation of birth weight and weaning weight is reported to be +.50.[9] With this fairly high positive correlation and the low accuracy numbers of natural service sires, one would assume that low birth weights are associated with lower weaning weights in the majority of bulls.

Intuition and experience make us want to believe the AI-sired calves will be heavier at weaning, but from the above data, we cannot automatically assume that the AI-sired calves will have a genetic advantage to be heavier at weaning.

Decreased dystocia rates in calves sired by high accuracy "calving ease bulls"

The higher Calving Ease Direct numbers of the high accuracy FTAI sires would indicate that likelihood of dystocia would be less in the FTAI group. The high accuracy numbers of the AI sires make the assumption much more valid. Dargatz et al reported in a survey of US beef producers that 16.7% of primaparous heifers suffered some degree of dystocia.[11] Dystocia rates in cattle significantly affect survivability of calves. In two studies at the U.S. Meat Animal Research Center (MARC), Clay Center, Nebraska, calf losses within 24 hours of birth averaged four percent for those born with little or no assistance compared with 16 percent for those requiring assistance. One report out of the Miles City, Montana research station showed that more than half (57%) of pre-weaning calf losses were due to dystocia.[12] Using these reports, a quantification of the value of decreasing dystocia can be calculated. If one assumes a pre-weaning increase in calf mortality due to dystocia of 12% (assisted = 16%- no assistance = 4%) and that we assume dystocia rates are decreased by using a high accuracy bull by 50%, that improvement in survivability would result in a 6% increase in number of calves sold (50% times

12%). If we assign a weaning value of $600 per calf the 6% increase in calves sold would return the producer $36 per pregnant heifer using FTAI.

The aforementioned MARC study found pregnancy rates 16% lower after a 70 day breeding season in cows that had been assisted at calving versus unassisted births (85% vs. 69%). Using the Dargetz et al data of 16.7% incidence of dystocia in primaparous heifers, we again assume using high accuracy sires in FTAI will reduce that dystocia incidence by 50% resulting in a dystocia rate in the FTAI group of 8.4%. By reducing the dystocia rate we would assume the subsequent pregnancy rate of that 8.4% would improve by 16% as in the MARC study. This 1.3% increase in overall pregnancy rate will bring about savings for the producer in the cost of culling open heifers. Again the cost of culling a first calf heifer versus retaining in the herd is $1160 per head. The increased rebreeding rate will save the producer $15.60 (.134 X $1160) per head in culling costs for heifers not rebreeding.

Conclusions

In this paper we conclude the following:

1. Partial budget spreadsheets allow producers and their veterinarians to compare the costs of producing bred heifers using either FTAI of 45 day natural service.
2. Survey results of cow-calf producers in Southwest Missouri reveal that, using their estimated costs of production, producing heifers bred to FTAI have an average advantage of $3.85 per pregnant female for FTAI over 45NS. The range of results was $174.22 disadvantage to $311.90 advantage for FTAI. Average cost of producing pregnant heifers using FTAI in this survey is $1445.87
3. Heifers pregnant by FTAI should have average calving dates at least 17 days earlier than those pregnant to natural service. These earlier calving dates should result in a $34 advantage per calf sold.
4. The earlier calving date of FTAI heifers will increase the calving to breeding interval by 17 days thus allowing more heifers to breed back in the breeding window. This effect helps establish some reproductive momentum in that group. Our calculations indicate that advantage is $104 per head.
5. Increasing weaning weights of FTAI calves due to genetic advantage is questionable. Data from natural service sires would indicate comparable weaning weights. It is noted that EPD accuracies would make the expected results more consistent.
6. A 50% reduction in dystocia incidence by using high accuracy FTAI sires would increase return to the producer by:
    a. Increased calf survival. We conclude that increased calf survival would allow the producer to sell 6% more calves. If calves are worth $600 each, then FTAI should increase return to the producer by $36 per head.
    b. Increased second calf pregnancy rates. Our study would indicate that a 50% decrease in dystocia would save the producer $15.60 per head in culling costs for heifers failing to get pregnant with a second calf due to complications from dystocia.

From this study we conclude that a heifer bred with FTAI to a high accuracy calving ease bull will cost no more and will return a producer an additional $189.60 per head compared to a heifer bred to a natural service sire in a short (45 day) season.

Data from 2003-2008 Sho-Me Select Heifer sales reveal average prices given by buyers for pregnant heifers. Heifers bred by AI sold for an average of $1290.88 while those pregnant to natural service $1211.68, a difference of $79.22.

Data from more recent Sho-Me Select heifer sales from the fall of 2010 through the fall 2012 revealed the Sho-Me Select heifers bred using AI sold for an average of $1830 per head while heifers with natural service sired pregnancies sold for an average of $1638 per head. The heifers pregnant by AI demanded a $192 per head premium.[13] Most of the heifers pregnant to AI in these sales were from FTAI breeding. The real world data would suggest that my conclusion of a $189.60 advantage for FTAI is close to what buyers in the marketplace believe also.

References

1. Blezinger SB: Artificial insemination: one of the industry's most underutilized tools. http://cattletoday.com/archive/2006/May/CT478.shtml.
2. Cupps D, Brown V: An enterprise partial budget analysis comparing costs of using fixed time artificial insemination and short season natural service breeding for breeding beef replacement heifers. Proc World Buaitrics Congr; 2012.
3. Larson RL: valueofimprovingpreghistogram.xls. Kansas State University, Beef Cattle Institute, beefcattleinstitute.org.
4. Reuter R, Robinette J: AI calving distribution. Ag News and Views, March 2010. https://www.noble.org/ag/livestock/aicalvingdistribution/.
5. Parish J: Reproductive management of beef cattle herds, Mississippi State Extension Bulletin Publication 2615. http://msucares.com/pubs/publications/p2615.pdf
6. Larson RL: A systematic approach to beef heifer development. Proc Acad Vet Consult; 2012.
7. Cole E: Personal communication. Area Livestock Extension Agent, University of Missouri Cooperative Extension Service, Mt. Vernon, MO.
8. ABS Global: Angus sire summary, Jan 2013. http://issuu.com/absglobalinc/docs/spring2013beefsdintl?mode=window&viewMode=doublePage.
9. Greiner SP: Genetic relationships. http://www.sites.ext.vt.edu/newsletter-archive/livestock/aps-01_10/aps-0408.html.
10. Short RE, Bellows RA, Staigmiller RB, et al: Physiological mechanisms controlling anestrus and infertility in postpartum beef cattle. J Anim Sci 1990:68:799-816.
11. Dargatz DA, Dewell GA, Mortimer RG: Calving and calving management of beef cows and heifers on cow–calf operations in the United States. Theriogenology 2004; 61:997-1007.
12. Ritchie H, Anderson PT: Calving difficulty in beef cattle: part 1. http://www.iowabeefcenter.org/Beef%20Cattle%20Handbook/Calving_Difficulty-1.pdf.
13. Patterson D: Personal communication. Professor of Animal Science, University of Missouri, Columbia, MO

Management factors that affect success of AI programs in beef cattle

G. Allen Bridges,[a] M.L. Day[b]
[a]North Central Research and Outreach Center, University of Minnesota, Grand Rapids, MN; [b]Department of Animal Sciences, The Ohio State University, Columbus, OH

Introduction

Numerous factors beyond selection of an estrous synchronization protocol influence the probability of pregnancy success in beef cattle. Any management practice that detrimentally affects the ability of a female to conceive early in the breeding season ultimately detracts from reproductive and production efficiency. Often, nutritional management is the factor that greatest influences reproductive competence in beef cattle. For beef heifers, proper nutrition during heifer development is essential to ensure timely sexual maturation and attainment of puberty. Failure to attain puberty prior to the initiation of an estrous synchronization protocol reduces a heifer's probability to conceive to artificial insemination (AI) and thus delays date at first calving. Following pubertal attainment, nutritional management is still critical as exposing females to nutritional stress immediately following AI can also result in decreased pregnancy rates. Therefore, proper nutritional management during heifer development and early gestation is critical for maximizing reproductive efficiency in beef operations.

Keywords: Nutrition, puberty, pregnancy rate, management

Nutrition and puberty in heifers

Age at puberty has an important impact on the productive, reproductive and economic efficiency of female cattle. The occurrence of this physiological event implies an adequate growth rate and development of the animal that supports the endocrinological mechanisms that lead to sexual maturity. Age at puberty and first conception will influence lifetime productivity of cattle.[1] In spite of the importance of this physiological endpoint, the metabolic signals that activate the endocrine mechanisms that trigger puberty are still unknown. In cattle, maturation of the reproductive axis occurs gradually as the animal grows towards mature size.

Post-weaning growth and age at puberty

Weight gain after weaning is a major variable that influences age and weight at puberty.[2] The influence of different diets during the post-weaning period on age of puberty has been extensively investigated. Growth rate between traditional weaning (six to eight months of age) and puberty was negatively associated with age at puberty.[3] Target weights at breeding have been developed to reflect these relationships and suggest that heifers should achieve a specific weight by the beginning of the breeding season in order to achieve high pregnancy rates.[4,5] It has been established that heifers should be fed to achieve 60 to 65% of their projected mature weight before their first breeding season to ensure high pregnancy rates.[4,6] Ferrell[7] demonstrated that heifers fed for a suboptimal average daily gain (ADG) tended to be older and lighter at puberty. More recently, Thallman et al[8] reported that across breeds, age and weight at puberty were approximately 357 d of age and 320 kg. Postweaning nutrition is a crucial determinant of age at puberty.

Preweaning growth rate and age at puberty

While less emphasis has been placed on the influence of preweaning growth rate on puberty, Wiltbank et al[9] demonstrated that the positive relationship of preweaning body weight (BW) gain and age at puberty, and indicated it was a more consistent predictor of age at puberty than postweaning gain. Others have also observed a reduction in age at puberty with increased weaning weight[10,11] and Roberts et al[12] found that age at puberty is affected more by growth rate pre-weaning and immediately postweaning, than growth rate just before breeding.

Altered preweaning nutrition and precocious puberty in heifers.

The influence of preweaning growth rate on age at puberty in heifers managed in a typical manner (i.e., weaned at approximately seven months of age) has been well established. However, several years ago we observed that a significant proportion of heifers attained puberty by seven to eight months of age when they were weaned early and fed a high concentrate diet at three to four months of age.[13] This observation led to the hypothesis that the reproductive axis could be precociously activated by feeding of a high concentrate diet beginning at approximately three months of age in beef heifers. We initiated a series of studies designed to assess the impact of diet, beginning after weaning at three to four months of age, on induction of precocious puberty (before 300 d of age), and the alterations in the reproductive axis that led to this premature event.

The primary objective of the first study (EXPT 1)[14] was to test the hypothesis stated above. To accomplish this, eighteen heifers were weaned at 73 ± 3 d of age (early weaned; EW) and 115 ± 3 kg of BW and fed one of two diets. The control (C) diet was fed to achieve BW gain (0.75 kg/d) similar to those that would have been achieved with their dams on pasture. The high concentrate (H) diet was formulated to support target BW gain of 1.5 kg/d. Diets resulted in BW gains shown in Figure 1. Realized ADG during the experimental phase of the study (99 to 286 d of age) was 1.27 ± 0.05 kg/d for the EWH and 0.85 ± 0.05 kg/d for the EWC treatment. Eight of nine heifers in the EWH treatment experienced precocious puberty vs. zero of nine heifers in the EWC treatment (Table 1; Figure 1).

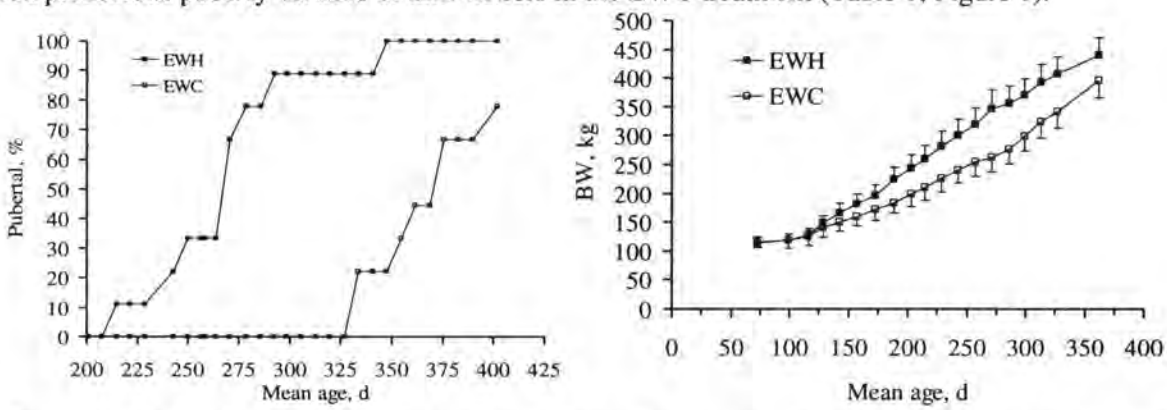


Figure 1. Body weight gain and cumulative percentage of heifers that attained puberty that were weaned early and fed a high concentrate diet (EWH) or control diet (EWC). Adapted from Gasser et al.[14].

Table 1. The percentage of heifers that experienced precocious puberty and age at puberty.*

		Early weaning, high concentrate diet (EWH)		Early weaning, control diet (EWC)	
Experiment	n	% Precocious puberty	Age at Puberty (d)	% Precocious puberty	Age at Puberty (d)
EXPT 1	18	89 (8/9)	262 ± 10	0 (0/9)	368 ± 10
EXPT 2	18	100 (9/9)	252 ± 9	56 (5/9)	308 ± 26
EXPT 3	10	80 (4/5)	275 ± 30	0 (0/5)	385 ± 14
EXPT 4	30	67 (10/15)	271 ± 17	20 (3/15)	331 ± 11

*Data from EXPT 1,[14] EXPT 2,[15] EXPT 3,[16] and EXPT 4.[17]

In the subsequent three experiments (EXPT 2, EXPT 3,EXPT 4)[15-17] similar differences in age at puberty (Table 1) were observed between heifers that received the EWC and EWH treatments, although the proportion of heifers that experienced precocious puberty varied somewhat among experiments. An

unexpected observation was that 56% of heifers in the EWC treatment in EXPT 2 experienced precocious puberty. Pasture feed resources preceding early weaning were excellent during this year and the heifers used in the experiment were approximately 35 kg heavier at approximately 100 d of age than heifers used in the other three studies. We speculate that the nutritional plane provided to these heifers during their first months of life before early weaning induced precocious puberty in some heifers. Indeed, spontaneous precocious puberty has been verified in a significant proportion of heifers weaned at 6 to 8 months of age.[18]

In EXPT 2[15] a treatment consisting of heifers that remained with their dams until weaning (208 d of age; normal weaning; NW) that were then fed the C diet postweaning (NWC treatment) was included to determine if age at weaning affected age at puberty directly. Average daily gain, age at puberty and incidence of precocious puberty did not differ between the EWC and NWC. Thus, in heifers growing at a typical and similar rate, large differences in timing of weaning and diet composition did not influence sexual maturation.

In EXPT 4[17] two treatments, in addition to the EWC and EWH were included to test the influence of the timing of feeding of the H diet on occurrence of precocious puberty. Diets were initiated at 126 d of age and the experiment was divided into Phase 1 (126 to 196 d of age) and Phase 2 (196 d of age +). Heifers in the EWC and EWH treatments received their indicated diets throughout both phases. Other heifers received the H diet for Phase 1 and the C diet for Phase 2 (EWH-C) while heifers in the fourth treatment received the opposite sequence (EWC-H). Body weight changes over the experiment are illustrated in Figure 2. Incidence of precocious puberty ranged from 67% in the EWH to 20% in the EWC treatment (Table 1). Age at puberty for the EWH and EWH-C treatments did not differ and was earlier than for the EWC treatment. Age at puberty in the EWC-H treatment was intermediate to and not different from the other treatments. Across treatments, heifers fed the H diet during Phase 1 attained puberty earlier than heifers fed the C diet during Phase 1. Increased dietary energy intake with the H diet from four to seven months of age advanced age at puberty; regardless of the diet fed after seven months of age.

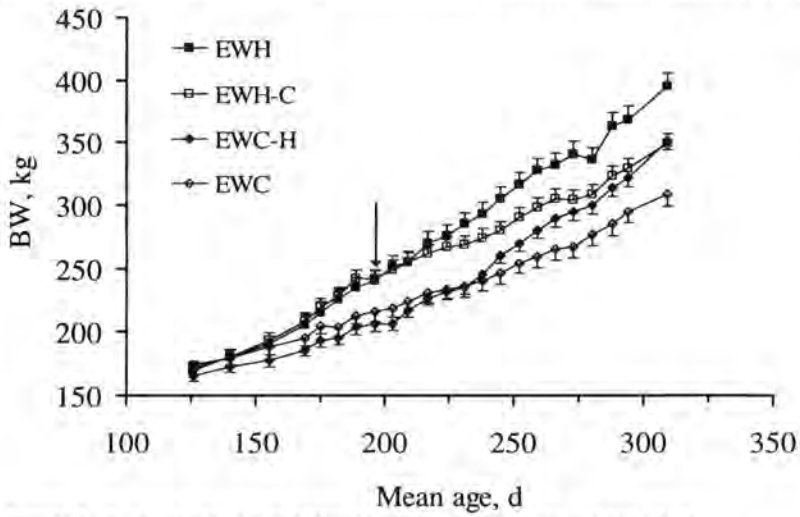


Figure 2. Body weight gain in heifers that were weaned early and fed a high concentrate diet (H) throughout (EWH), H diet Phase 1 and C diet Phase 2 (EWH-C), C diet Phase 1 and H diet Phase 2 (EWC-H) or the C diet throughout (EWC) in Phase 1 (126 to 196 d) and Phase 2 196 to 325 d). Adapted from Gasser et al., 2006d.

Based upon these and other experiments, feeding a high energy diet to heifers that are weaned at three to four months of age induces precocious puberty in most heifers. Precocious puberty occurred sporadically with the control diet appeared to be related to the nutritional status of the heifer calves preceding early weaning. Consistent with this speculation, feeding of the high energy diet from early weaning to approximately 200 d of age had a similar impact on age at puberty to feeding this diet through 11 to 12 months of age. Hence, the signal that induces precocious activation of the reproductive axis may appears to occur early in life of heifers, suggesting existence of a critical time in which nutritional management can influence age at puberty. Thus, nutritional management throughout the period from birth to the first breeding season in heifers can ultimately alter breeding success in this season.

Post-insemination nutrition and fertility in beef heifers

Following pubertal attainment, reproductive competence can still be influenced by nutritional inputs. Recently, the role of nutritional inputs in beef heifers following AI on the probability of

conception has been investigated. Many spring-born heifers are developed from weaning to breeding in a dry-lot scenario and fed a diet consisting of a combination of forage and concentrate needed to gain approximately 1.5 lb. per day, targeting a final weight of 65% of estimated mature body weight at the time of breeding. Often estrus is synchronized and AI is conducted while in the dry-lot to better facilitate protocol implementation. Immediately following AI, heifers are often moved to pastures to expose them to clean-up bulls, take advantage of lush spring forage, and reduce the incidence of embryonic loss associated with handling and moving animals at later stages of early gestation. Such an immediate change in nutrition, due to shift in diet delivery method and/or quality and quantity of nutrients, may negatively impact metabolism, body weight gains, and reproductive efficiency in these heifers.

During early embryonic development, the developing conceptus is completely dependent upon secretions from the uterine endometrium, termed histotroph, to supply the nutrients necessary for conceptus growth and survival.[19] Uterine histotroph is comprised of enzymes, cytokines, growth factors, ions, hormones, glucose, fructose, amino acids, transport proteins, and adhesion molecules.[20,21] Many factors that are associated with metabolic status of an animal when measured in general circulation are also present in the histotroph during early gestation.[22-26] Thus, an immediate shift in nutritional intake and an alteration in metabolic status may perturb uterine function and compromise embryonic development and maintenance of pregnancy.

Concerns about growth and reproductive performance of beef heifers developed in a dry-lot scenario and then immediately placed on spring pastures following estrous synchronization and AI led to the initial investigations of post-AI nutrition on pregnancy success in beef heifers.[27] The initial objectives of these series of investigations were to assess how heifer development strategy, dry-lot versus range development, impacted growth parameters and pregnancy success in beef heifers that were placed on pasture at the initiation of the breeding season. It was demonstrated that heifers developed in the feedlot and then placed on pasture lost considerable weight in the first week after placement on pasture (Figure 3). Furthermore, it was demonstrated that developing heifers in a dry-lot (n = 92) and then moving them to spring pastures immediately after estrous synchronization and AI resulted in a tendency ($P = 0.0108$; 57% vs. 45%) for reduced AI pregnancy rates compared to when heifers were developed on range (n = 91) throughout.[27] It is likely that the abrupt shift in metabolic status of heifers moved from the dry-lot to pastures is directly responsible for this reduction in pregnancy success. In a subsequent experiment, supplementing heifers developed in a dry-lot with distiller's grains (supplement; 2.27 kg/day; 76%) when placed on pasture after AI, prevented a decrease in AI pregnancy rates, as was observed if heifers were not supplemented (61%) following AI. Thus, if heifers were provided a high-energy and protein supplement once introduced to pastures, pregnancy rates were improved, likely because weight loss was mitigated and heifers did not perceive a drastic shift in metabolic status.

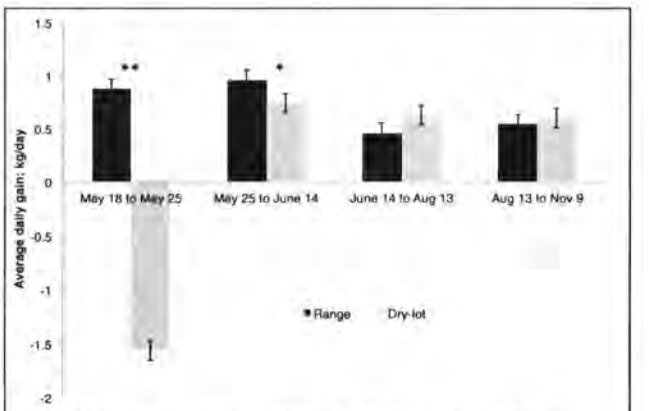


Figure 3. Average daily gains (kg/day) of heifers weaned and developed on range (Range) compared to heifers weaned and developed in a dry-lot (Dry-lot). All heifers were moved to a common pasture on May 18th. $^{*}P = 0.06$, $^{**}P < 0.05$

Further investigations of the influence on post-AI nutrition on pregnancy success in beef heifers have been conducted.[28] This study was conducted over two years (2011 and 2012) at two locations per year (total of four replications), heifers were developed and maintained in a dry-lot scenario. Prior to AI, heifers were fed to achieve body weight gains to reach 65% of mature body weight at the start of the breeding season. Following estrous synchronization and on the day of AI heifers were allotted to one of three post-insemination treatments to which heifers remained for 21 days. Post-insemination dietary treatments were: 1) 120% NEm (Gain), 2) 100% NEm (Maintain), and 3) 80% NEm (Lose). During the treatment period, dietary components were similar but NEm targets were achieved through limiting feed

delivery. Although AI pregnancy rates did not differ between treatments (Table 2), contrast analyses reveled that AI pregnancy rates were reduced ($P = 0.05$) in the treatments where NEm of diets were not adequate for continued growth (Lose and Maintain) compared to the Gain treatment. Likewise, when assessed via contrast analyses, breeding season pregnancy rates tended ($P = 0.106$) to be reduced in treatments where diets did not allow post-insemination body weight gains (Lose and Maintain) compared to the Gain treatment (Table 2). Collectively, these data strongly suggest that nutrient intake and body weight changes following insemination influences the probability of pregnancy.

Table 2. AI and breeding season pregnancy rates in beef heifers fed to 120% (Gain), 100% (Maintain), and 80% (Lose) NEm following insemination.

	Treatment (Trt)				*P*-value	
	Gain 120% NEm	Maintain 100% NEm	Lose 80% NEm	Trt	Contrast: Gain vs Maintain + Lose	Contrast: Maintain vs Lose
AI pregnancy rates,† % (n)	72.9% (86/118)	62.3% (71/114)	64.7% (75/116)	0.13	0.05	0.73
Breeding season pregnancy rates,‡ % (n)	94.1% (111/118)	87.7 (100/114)	88.8 (103/116)	0.24	0.106	0.69

†Treatment x Replication, $P = 0.39$, thus replications combined for analyses.
‡Treatment x Replication, $P = 0.65$, thus replications combined for analyses.

Based on timing of nutritional insult relative to fertilization, it is likely that pregnancy failure previously observed is not due to inherit alterations in oocyte quality but rather nutritionally-induced alterations in uterine function that are augmenting normal embryonic development. Recently, we determined if post-insemination nutrient restriction directly impacted early embryo quality and the number of live/dead blastomeres.[29] The study was conducted at two locations, University of Minnesota's North Central Research and Outreach Center (UMN; two replications) and South Dakota State University (SDSU; one replication). All heifers were on a common diet during development. Estrus was synchronized and timed-AI was conducted. On the day of AI, heifers were placed in one of two nutritional treatments. Heifers either continued on the pre-insemination diet (~120% NEm; GAIN) or were fed a sub-maintenance diet (LOSE; UMN, 80% NEm; SDSU, 50% NEm). Dietary treatments were fed until single embryos were collected using non-surgical embryo flush techniques six days after AI. Recovered embryos were microscopically evaluated, classified by developmental stage and graded (per International Embryo Transfer Society standards) and then the number of dead blastomeres and total number of blastomeres was evaluated using epifluorescent staining (Table 3). Visual embryo assessment by a trained embryologist blind to treatments revealed that embryo stage was less ($P < 0.05$) and embryo quality was decreased ($P < 0.05$) in the LOSE compared to GAIN treatment. In accordance with embryo stage results, total number of blastomeres within embryos in the LOSE treatment was decreased ($P < 0.05$) compared to embryos in the GAIN treatment. Although the number of dead cells did not differ between treatments, proportion of live cells within embryos was tended ($P < 0.10$) to be decreased in the LOSE compared to the GAIN treatment. Results indicated that embryo development in heifers receiving insufficient energy intakes following insemination was retarded within six days of AI.

In summary, 1) beef heifers developed in dry-lots, although receiving a primarily forage-based diet during development, experience considerable weight loss when introduced to pastures in the spring; which likely results in metabolic alterations to cope with this alteration in homeostasis, 2) an abrupt change in nutrient intake immediately following AI and delivery of post-insemination diets that do not exceed NEm requirements results in a reduction of AI pregnancy rates, and 3) immediate alterations in early embryonic development are observed in heifers that fail to receive adequate nutritional inputs following insemination and these alterations in embryonic development is likely due to insufficient oviductal and uterine support of the developing embryo.

Overall summary

Nutritional management of heifer prior to and following the start of the breeding season can influence reproductive efficiency. Rate of sexual maturation and timing of puberty attainment is greatly influenced by nutrition. Although post-weaning nutritional management is often the focus of most attention, pre-weaning nutrition likely plays an equal or greater role in age at puberty in beef heifers. Nutritional management immediately after AI appears to also influence the probability of pregnancy success. Invoking an immediate shift in nutritional inputs following AI, during early gestation, alters embryonic development and has the potential to reduce AI pregnancy rates. Thus, nutritional management of the beef heifer prior to and immediately following AI is essential to ensure maximum reproductive success.

Table 3. Effect of post-AI nutrition on day 6 embryo development in which heifers were either fed 125% of NRC requirements (GAIN) or below maintenance (LOSE) immediately following AI

TRT	n[a]	% Embryos Recovery	Embryo Stage[b]	Embryo Quality[c]	Accessory Sperm (n)	Dead Cells (n)	Total Cells (n)	% Live Cells
GAIN	44	67.7 (44/65)	4.4 ± 0.16	2.2 ± 0.19	19.9 ± 3.93	7.9 ± 1.04	66.9 ± 5.05	80.9 ± 4.19
LOSE	41	62.1 (41/66)	3.7 ± 0.16	2.9 ± 0.19	15.4 ± 3.99	9.5 ± 1.11	47.9 ± 5.41	69.7 ± 4.39
P-value	.	.	< 0.005	< 0.05	NS	NS	< 0.01	< 0.10

[a] Defined as embryo number; not heifer with the exception of recovery rate
[b] Stage of development (1-9;1 = UFO; 9 = expanded hatched blastocyst; per IETS Standards)
[c] Quality of embryo (1-5;1 = excellent; 5 = degenerate; per IETS Standards)

References

1. Bagley CP: Nutritional management of replacement beef heifers: a review. J Anim Sci 1993;71:3155-3163.
2. Patterson DJ, Perry RC, Kiracofe GH, et al: Management considerations in heifer development and puberty. J Anim Sci 1992;70: 4018-4035.
3. Smith GM, Fitzhugh HA Jr, Cundiff LV, et al: A genetic analysis of maturing patterns in straightbred and crossbred Hereford, Angus and Shorthorn cattle. J Anim Sci 1976;43:389-395.
4. Lamond DR: The influence of undernutrition on reproduction in the cow. Anim Breed Abst 1970;38:359-372.
5. Wiltbank JN, Kasson CW, Ingalls JE: Puberty in crossbred and straightbred beef heifers on two levels of feed. J Anim Sci 1969;29:602-605.
6. Taylor CS, Fitzhugh HA Jr: Genetic relationships between mature weight and time taken to mature within a breed. J Anim Sci 1971;33:726-731.
7. Ferrell CL: Effects of postweaning rate of gain on onset of puberty and productive performance of heifers of different breeds. J Anim Sci 1982;55:1272-1283.
8. Thallman RM, Cundiff LV, Gregory KE, et al: Germplasm evaluation in beef cattle--Cycle IV: postweaning growth and puberty of heifers. J Anim Sci 1999;77:2651-2659.
9. Wiltbank JN, Gregory KE, Swiger LA, et al: Effects of heterosis on age and weight at puberty in beef heifers. J Anim Sci 1966;25:744-751.
10. Arije GF, Wiltbank JN: Age and weight at puberty in Hereford heifers. J Anim Sci 1971;33:401-406.
11. Greer RC, Whitman RW, Staigmiller RB, et al: Estimating the impact of management decisions on the occurrence of puberty in beef heifers. J Anim Sci 1983;56:30-39.
12. Roberts AJ, Geary TW, Grings EE, et al: Reproductive performance of heifers offered ad libitum or restricted access to feed for a one hundred forty-day period after weaning. J Anim Sci 2009;87:3043-3052.
13. Day ML, Anderson LH: Current concepts on the control of puberty in cattle. J Anim Sci 1998;76 (Suppl 3):1-15.
14. Gasser CL, Grum DE, Mussard ML, et al: Induction of precocious puberty in heifers I: enhanced secretion of luteinizing hormone. J Anim Sci 2006;84:2035-2041.
15. Gasser CL, Burke CR, Mussard ML, et al: Induction of precocious puberty in heifers II: advanced ovarian follicular development. J Anim Sci 2006;84:2042-2049.

16. Gasser CL, Bridges GA, Mussard ML, et al: Induction of precocious puberty in heifers III: Hastened of estradiol negative feedback on secretion of luteinizing hormone. J Anim Sci 2006;84:2050-2056.
17. Gasser CL, Behlke EJ, Grum DE, et al: ML. Effect of timing of feeding high-concentrate diet on growth and attainment of puberty in early-weaned heifers. J Anim Sci 2006;84:3118-3122.
18. Wehrman ME, Kojima FN, Sanchez T, et al: Incidence of precocious puberty in developing beef heifers. J Anim Sci 1996;74:2462-2467.
19. Bazer FW: Uterine protein secretions: relationship to development of the conceptus. J Anim Sci 1975;41:1376-1382.
20. Bazer FW, Song G, Kim J, et al: Mechanistic mammalian target of rapamycin (MTOR) cell signaling: effects of select nutrients and secreted phosphoprotein 1 on development of mammalian conceptuses. Mol Cell Endocrinol 2012;354:22-33.
21. Mullen M, Elia G, Hilliard M, et al: Proteomic characterization of histotroph during the preimplantation phase of the estrous cycle in cattle. J Proteome Res 2012;11:3004-3018.
22. Gao H, Wu G, Spencer TE, et al: Select nutrients in the ovine uterine lumen. I. Amino acids, glucose, and ions in uterine lumenal flushings of cyclic and pregnant ewes. Biol Reprod 2009;80:86-93.
23. Gao H, Wu G, Spencer TE, et al: Select nutrients in the ovine uterine lumen. II. Glucose transporters in the uterus and peri-implantation conceptuses. Biol Reprod 2009;80:94-104.
24. Gao H, Wu G, Spencer TE, et al: Select nutrients in the ovine uterine lumen. III. Cationic amino acid transporters in the ovine uterus and peri-implantation conceptuses. Biol Reprod 2009;80:602-609.
25. Gao H, Wu G, Spencer TE, et al: Select nutrients in the ovine uterine lumen. IV. Expression of neutral and acidic amino acid transporters in ovine uteri and peri-implantation conceptuses. Biol Reprod 2009;80:1196-1208.
26. Gao H, Wu G, Spencer TE, et al: Select nutrients in the ovine uterine lumen. V. Nitric oxide synthase, GTP cyclohydrolase, and ornithine decarboxylase in ovine uteri and peri-implantation conceptuses. Biol Reprod 2009;81:67-76.
27. Perry GA, Walker J, Wright C, et al: Impact of method of heifer development and post-AI management on reproductive efficiency. Proc Range Beef Cow Symp XXI, Casper WY.
28. Arias RP, Gunn PJ, Lemenager RP, et al: Effects of post-AI nutrition on reproductive and growth performance of yearling beef heifers (abstract). J Anim Sci 2012;90(Suppl 3);156.
29. Kruse SG, Funnell BJ, Bird SL, et al: Influence of post-insemination nutrition on embryonic development in beef heifers. J Anim Sci 2013; (to be presented at 2013 Natl Am Soc Anim Sci Meet, Indianapolis, IN.

Estrus synchronization in goats

Lionel J. Dawson
Boren Veterinary Medical Teaching Hospital, Oklahoma State University, Stillwater, OK

Abstract

Estrus synchronization allows breeding does in a short period of time by effectively using the bucks, or by performing artificial insemination (AI). Synchronization of the does will allow increased proportion of does becoming pregnant in a short period of time, uniform size of kids at weaning, take advantage of the niche in the market for meat during religious events and rising price trends in the market. In the past synchronization of estrus in goats has focused primarily on dairy goats to allow for optimal timing of milk production. Methods of synchronization have included techniques such as alteration of light patterns, manipulation of social interaction, buck exposure early in the breeding season and manipulating the estrous cycle by extending or shortening the luteal phase of the cycle. During the breeding season the opportunity to control the estrous cycle is greater during the luteal phase which is of longer in duration and is more responsive to manipulation.

Keywords: Synchronization, estrous cycle, manipulation

Introduction

Goats are generally classified as a seasonally polyestrous or short day breeders in the U.S. The degree of seasonally varies among breeds and their locations (latitude). The annual reproductive cycle of goats in a temperate region can be divided into the breeding season or period, the nonbreeding season or physiologic anestrous period and the transitional period. The transitional period is between the nonbreeding and the breeding season.

Estrus synchronization allows breeding does in a short period of time by effectively using the bucks, or improving the genetic make-up of the flock by performing AI. With the rapid increase in meat goats in the U.S., estrus synchronization has been used as an effective tool in the reproductive management of these herds. Synchronization early in the breeding season will allow an increased proportion of does becoming pregnant early, older and uniform size of kids at weaning, take advantage of the niche in the market during religious events and rising price trends in the market.

In the past synchronization of estrus in goats has focused primarily on dairy goats to allow for optimal timing of milk production. Methods of synchronization have included techniques such as alteration of light patterns, manipulation of social interaction like buck exposure early in the breeding season and manipulating the estrous cycle by extending or shortening the luteal phase of the cycle. During the breeding season the opportunity to control the estrous cycle is greater during the luteal phase which is of longer in duration and is more responsive to manipulation. Strategies can be employed for synchronization, is to extend the luteal phase by supplying exogenous progesterone or to shorten this phase by prematurely regressing the existing corpus luteum (CL) by using prostaglandin.[1-7] Hormones have been used in goats to manipulate the estrous cycle, but none have been approved for use in goats in the U.S.

Discussion

Extending the luteal by supplying exogenous progesterone is best done by using controlled internal drug release devices (CIDR), intravaginal sponges and feed supplements.[2] Regressing or lysing the CL is best done by utilizing prostaglandin. Progesterone or progestagens products commonly used are CIDR's and sponges (Veramix, Repromap, Sincrocel, Cronolone and Chronogest). Shortening the luteal phase is best done by using dinoprost tromethamine and cloprostenol.[1-7]

For better control or synchrony of estrus and ovulation, extending the luteal phase with progesterone, along with a gonadotropin (follicle stimulating hormone; FSH) and prostaglandin have been used. The gonadotropin commonly used is equine chorionic gonadotropin (eCG) because of its longer half-life. The drawback of using higher doses eCG may result in a larger number of anovulatory follicles

and repeated doses can cause declining fertility due to the buildup of antibodies against eCG.[7-8] Equine chorionic gonadotropin is not commercially available in the U.S. but a product containing eCG and human chronic gonadotropin (hCG) which has been labeled to be used in swine (PG 600; Intervet Inc, Merck Animal Health, Summit, NJ) for induction estrus in gilts has been tried and used in goats. The dosage of PG 600 utilized in does is 200 units of eCG + 100 units of hCG (1/2 dose) during the breeding season or 400 units of eCG + 200 units of hCG (full dose) during the non-breeding or off season. If eCG is used, the dose is 200 units of eCG during the breeding season and 400 units of eCG during the non-breeding or off season.

During the transitional period (late July to early September), buck effect is a powerful tool to induce estrus (Table1). Sudden introduction of previously isolated bucks will stimulate a surge of luteinizing hormone (LH) followed by ovulation and majority of the does exhibit estrus within 48-72 hours.[1,3,9-11] Thirty to 60% of does will show estrus behavior and ovulate within 3-5 days or 7-12 days after introduction of the buck. Three peaks of estrus activity have been observed, after the introduction of the buck, 3-5 days, 7-12 days and 28 to 35 days after introduction to the buck.[9] This phenomenon of cycling in seven to 12 days is described as early luteal regression (ELR).[3,4,7-14] The current thinking is that ELR maybe due to lack of progesterone priming of the uterus during the anestrus and early transition periods. Estrogen produced by the follicles at that time will have a positive effect on the uterus. Estrogen increases the availability of oxytocin receptors leading to the release of endogenous prostaglandin and thereby lysing the CL. Does that were supplemented with progesterone in late-transition which was removed on the day the bucks were introduced had higher percentage showing estrus and reduced the number of short cycles. Buck effect and exogenous progesterone are methods commonly employed to induce estrus in the transition period and to prevent ELR (Table1).[12-14]

Out of season breeding in does could be done by using hormones or manipulating the photoperiod to hasten the onset of estrus. Out of season breeding will enable the producer to take their kid crop to market when prices are higher, have year round milk production in dairy animals and also increase the number of kids born to the doe during her lifetime. Hormones are commonly used effectively to synchronize estrus in this period.

Incorporating FSH into the protocol is essential to stimulate follicular waves during the non-breeding season or off season. Equine chorionic gonadotropin is commonly used in the U.S. PG 600 which contains eCG is available in the U.S. and has been used successfully used in goats (Table 2).

Photoperiod manipulation is done by altering the day length. Decreasing day length will increase the levels of melatonin produced by the pineal gland. Melatonin production may influence the secretion of LH from the anterior pituitary gland and hasten cyclicity.[25-38] Melatonin will increase the pulsatile release of gonadotropin releasing hormone (GnRH), thereby increasing the frequencies of FSH and LH release. Increased levels of LH release will cause ovulation, and thus enabling doe to cycle regularly. Change in light exposure or decreasing day length requires at least 45-60 days to induce a doe to cycle. Gradual change is not necessary; the amount of change that is perceived by the eye is important. A reduction of light is effective to trigger estrus activity in 30 to 60 days in a doe.[19,24,39,40]

Exogenous melatonin can be administered to supplement endogenous release and thereby mimic short days associated with the breeding season.[19,24,39,40] Melatonin is more effective in advancing the breeding season than inducing or initiating it and is more effective if melatonin is given after the doe is exposed to long days.

There are numerous protocols for synchronizing the estrous cycle in a doe have been used and described in the literature. Protocols 1 and 2 have been utilized by the author in various clinical trials at the American Institute for Goat Research at Langston University. For AI involving transcervical or laparoscopic insemination protocol 2 with timed AI has been used with good success. Dr. Nutti, a reproductive physiologist at Prairie View A&M, has utilized protocols 4 to 6 in conducting AI workshops in the U.S. and other countries where different pharmaceutical agents were not readily available. Prostaglandin by itself is used primarily during the breeding season to synchronize estrus in does.

Table 1.

Methods employed to hasten estrus activity during the transition period		
Method	**Duration**	**Estrous**
1.Buck effect	Late transition	24-96 hours
2. Progesterone(12-14 days) + equine chorionic gonadotropin(eCG) on the day or 24-48 hours prior to removal	Early transition	24-72 hours
3. Progesterone(12-14days) + eCG on the day or 24 to 48 hours prior to removal	Late transition	24-48 hours
4. Progesterone(10days) +eCG(removal) +prostaglandin 48 hours prior to removal	Late transition	40.9+- 3.2 hours

This table is adapted from Current therapy in large animal theriogenology

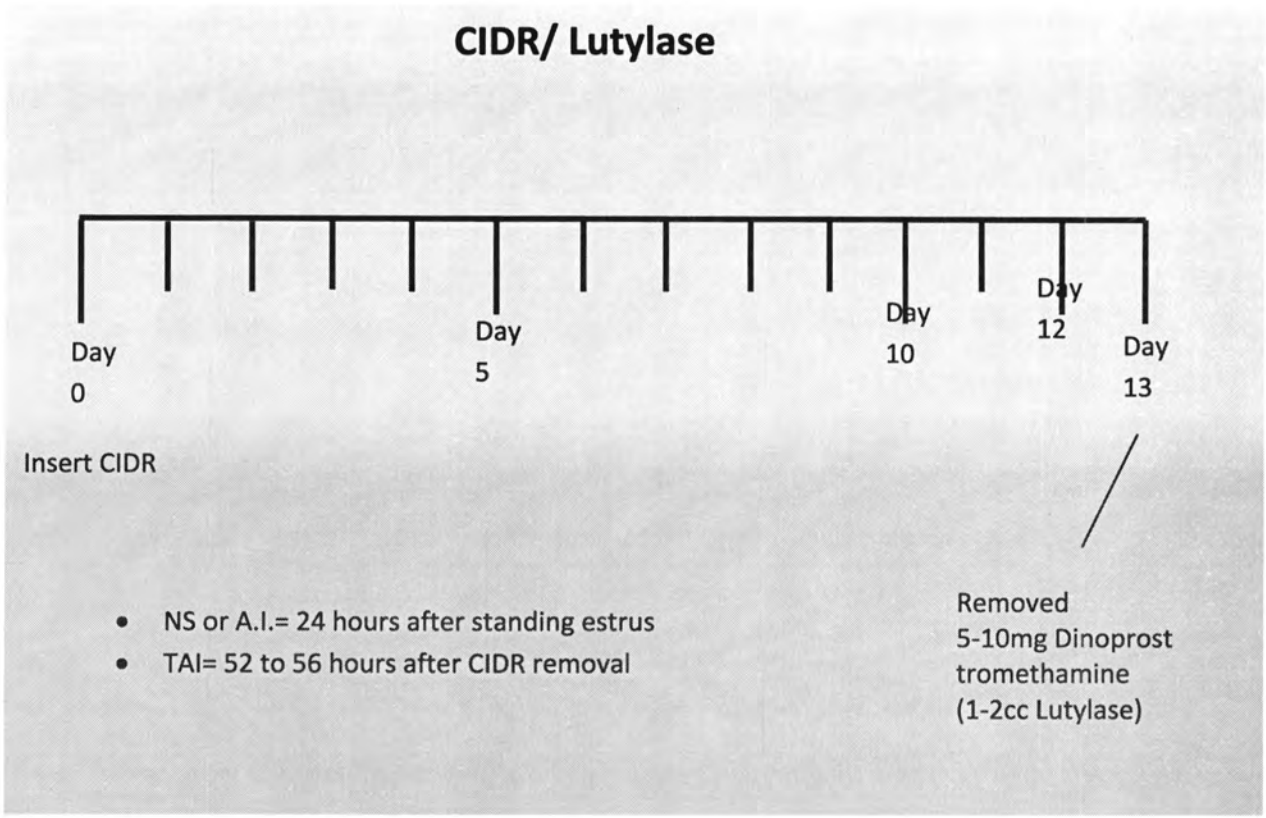
Table 2.

Methods employed during the non-breeding season

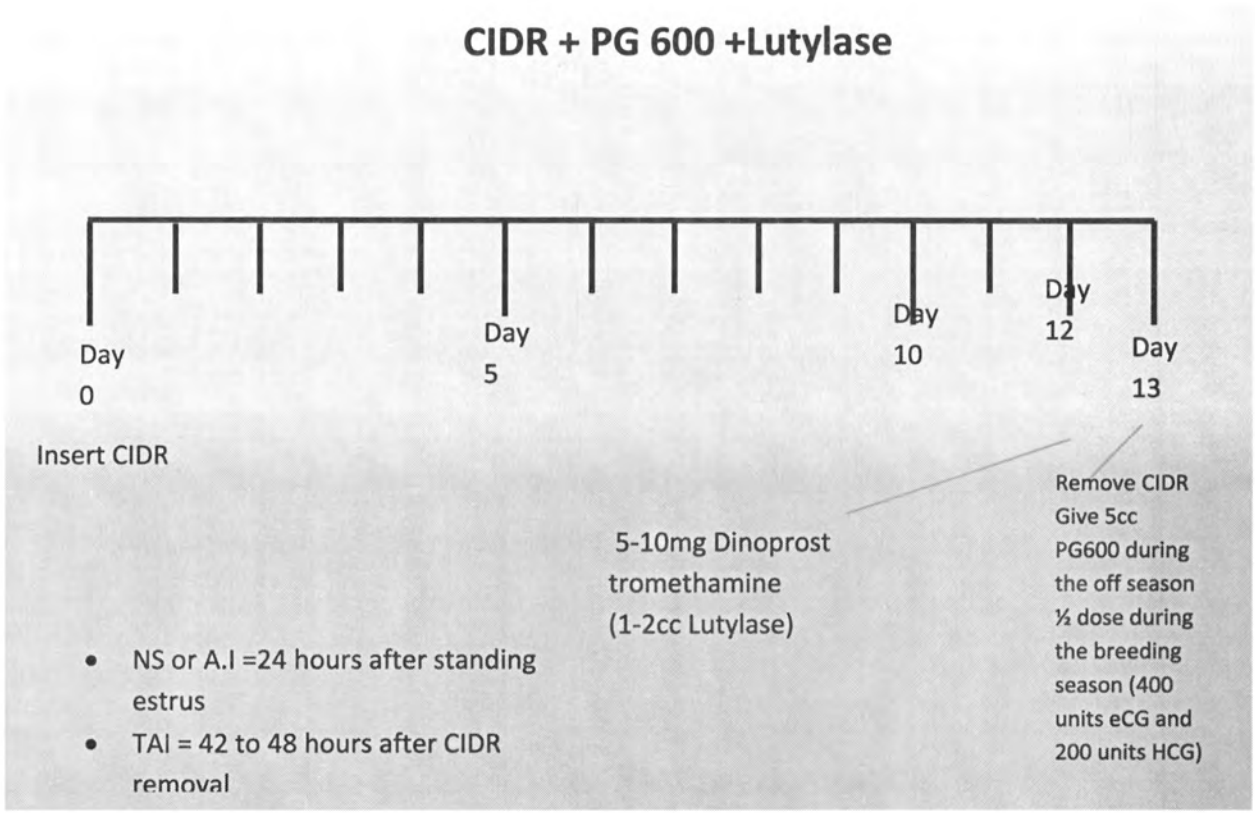
Method	Duration	Estrus
Progesterone	12-14 days	24-96 hours[1,2,3]
+Equine Chorionic Gonadotropin	On the day of removal or 24-48 hours before	44.6 ± 8.2 hours[15]
+Prostaglandin	On the day of removal or 24-48 hours before	25 ± 5 hours[8,16-18] <72 hours - 90% in estrus
Artificial lights	Mimic long days for 60 days followed by short days for 60 days or natural light	40-70 days during short days[19]
Melatonin (Implant, oral, or injection)	60-100 days	30-60 days[40]
Artificial lights + melatonin	Mimic long days for 60 days followed by melatonin for 60-100 days	Buck exposure 60 days into melatonin treatment; estrus 2-3 days[24]

Common synchronization of protocols used in does

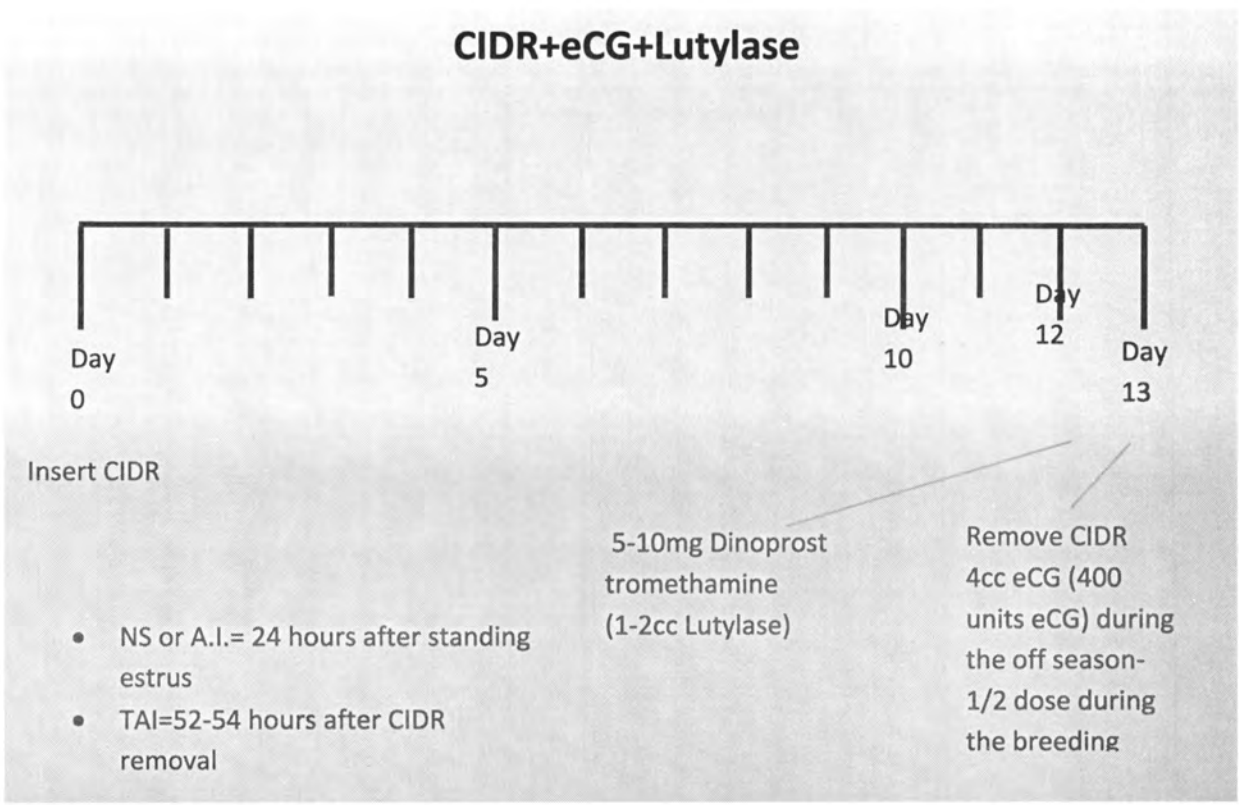
1.


CIDR/ Lutylase
Day 0
Day 5
Day 10
Day 12
Day 13
Insert CIDR
Removed
5-10mg Dinoprost tromethamine
(1-2cc Lutylase)
• NS or A.I.= 24 hours after standing estrus
• TAI= 52 to 56 hours after CIDR removal

2.


CIDR + PG 600 +Lutylase
Day 0
Day 5
Day 10
Day 12
Day 13
Insert CIDR
5-10mg Dinoprost tromethamine (1-2cc Lutylase)
Remove CIDR Give 5cc PG600 during the off season ½ dose during the breeding season (400 units eCG and 200 units HCG)
NS or A.I =24 hours after standing estrus
TAI = 42 to 48 hours after CIDR removal

3.


CIDR+eCG+Lutylase
Day 0
Day 5
Day 10
Day 12
Day 13
Insert CIDR
5-10mg Dinoprost tromethamine (1-2cc Lutylase)
Remove CIDR 4cc eCG (400 units eCG) during the off season- 1/2 dose during the breeding
NS or A.I.= 24 hours after standing estrus
TAI=52-54 hours after CIDR removal

4. Select Synch & CIDR

Day 0	Day 7	Day 7-12
➢ GnRH ➢ CIDR(in)	➢ PGF2 ➢ Remove CIDR	➢ HD & AI

5. Select Synch/CIDR/TAI

Day 0	Day 7	Day 10
➢ CIDR (in) + GnRH	➢ CIDR(out) + PGF	➢ TAI at 72 hours

6. Select Synch/CIDR/TAI/GnRH

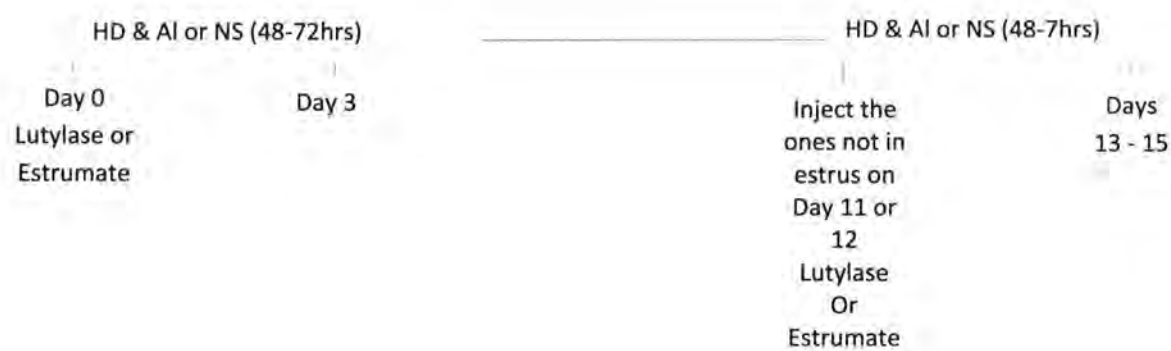
Day 0	Day 7	Day 7-10	Day 10
➢ GnRH ➢ CIDR	➢ PGF2 ➢ Remove CIDR	➢ HD & AI for 72 hrs	72 hours TAI & GnRH

GnRH-50 micrograms

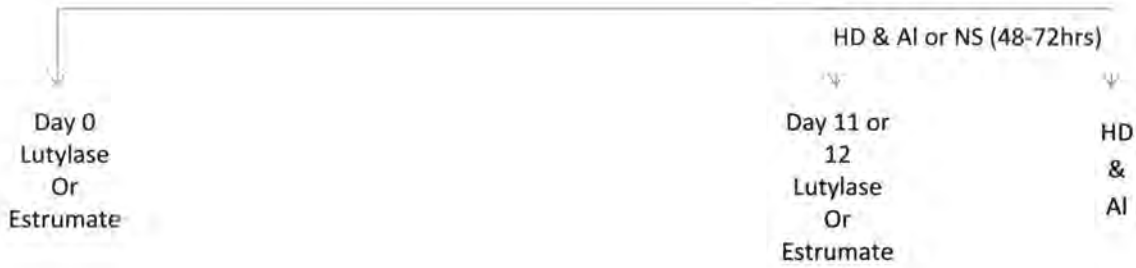
7. Shortening the luteal phase

Product	Dosage	Treatment	Route	Estrus
Lutalyse (Dinoprost tromethamine)	5-10 milligrams	2 injections, 11 to 12 days apart in does	I/M	24-72 hours (48-60 hours) TAI + 50-52 hours
Estrumate (Cloroprostenol)	50-150 micrograms	2 injections, 11 to 12 days apart	I/M	24-72 hours (48-60 hours) TAI=50-52 hours

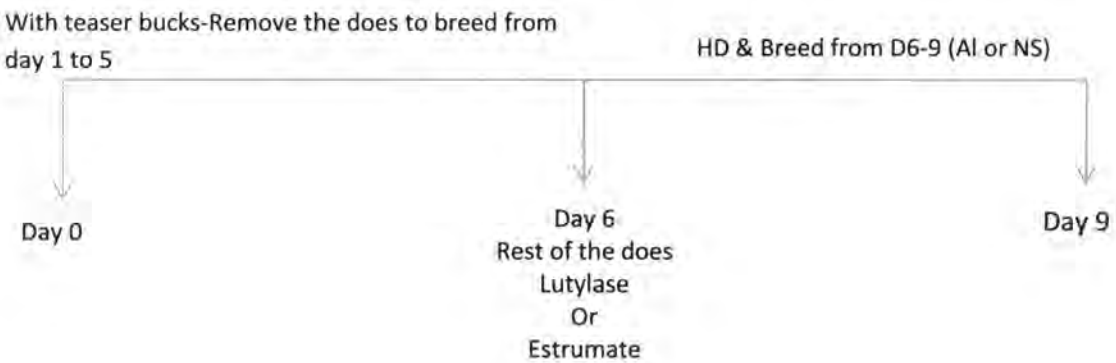
8. Two injections of prostaglandin = Heat detection two periods



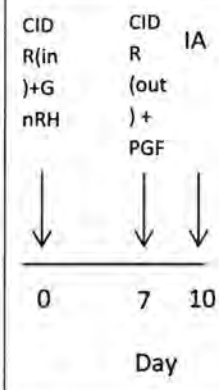
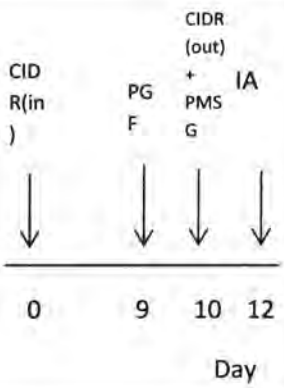
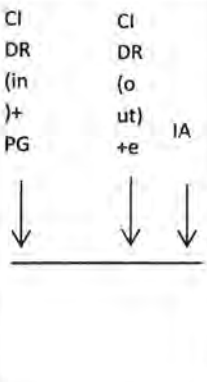
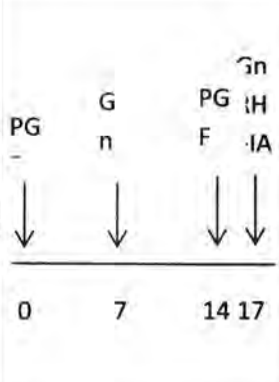
9. Two injections of prostaglandin = Heat detection one period



10. Heat detection and prostaglandin



11.

Event	Prairie View A&M	Langston #1	Langston #2	North Carolina
CIDR	In for 7 days	In for 10-14 days	In for 6 days	Not used
Prostaglandin (Lutalyse)	1 ml given 3 days before anticipated AI and at time of pulling CIDRs	2ml given 24 hours before pulling CIDRs	2 ml given with placing of CIDRs	2 ml given at days 0 and 14
GnRH(Cystorelin)	1 ml given with placing of CIDRs	Not used	Not used	1 ml given at days 7 and 17
PMSG/eCG(PG-600)	Not used	2.5 ml(half-dose) given at the time of pulling CIDRs	2.5ml (half dose) given at the time of pulling CIDRs	Not used
Diagram				

Summary

There has been rapid progress during the last decade in the U.S. toward manipulating the estrus cycle in does. With the availability of CIDRs in the U.S and approval for use in sheep, data have been collected by Iowa State University regarding approval for use in goats. Prostaglandin, PG 600, GnRH and CIDRs are not approved for use in goats. They have been used in an extra-label manner and the practitioner should be aware that under AMUDUCA regulations non-therapeutic extra-label use is not permitted. Thus regulatory guidance should be requested in use of these products in goats.

References

1. Dawson LJ: Manipulating the estrous cycle in a doe. In: Youngquist RS, Threlfall WR, editors. Current therapy in large animal theriogenology. 2nd ed. St. Louis: Saunders; 2007. p. 540-546.
2. Wildeus S: Current concepts in synchronization of estrus: sheep and goats. Proc Am Soc Anim Sci; 1999-2000. p. 1-9.
3. Whitley NC, Jackson DJ: An update on estrus synchronization in goats: a minor species. J Anim Sci 2004;82(E Suppl):E270-276.
4. Bretzlaff K: Control of the estrous cycle. In: Youngquist RS, editor. Current therapy in large animal theriogenology. Philadelphia: Saunders; 1997. p. 510-514.

5. Keisler DH, Buckrell BC: Breeding strategies in ewes. In Youngquist RS, editor. Current therapy in large animal theriogenology. Philadelphia: Saunders; 1997. p. 603-611.
6. Buckrell BC: Hormonal management of breeding in sheep and goats. Proc Small Ruminant Short Course (ACT and SFT); 1994. p. 32-40.
7. Pugh DG: Female reproduction in the ewe and doe. In: Sheep and goat medicine. Philadelphia: Saunders; 2002. p. 147
8. Baril G, Remy B, Leboeuf B, et al: Synchronization of estrus in goats. The relationship between eCG binding in plasma, time of occurrence of estrus and forcibly following artificial insemination. Theriogenology 1996;45:1553-1559.
9. Chimineau P: Possibilities for using bucks to stimulate ovarian and estrous cycles in anovulatory goats–a review. Livestock Prod Sci 1987;17:135-147.
10. Ott RS, Nelson DR, Hixon JE: Effect of presence of the male on initiation of estrus cycle activity of goats. Theriogenology 1980; 13:183-190.
11. Mellado M, Olivas R, Ruiz F: Effect of buck stimulus on mature and pre-pubertal norgestomet treated goats. Small Rum Res 2000;36:269-274.
12. Beard AP, Hunter MG: Effects of bovine follicular fluid and exogenous estradiol on the GnRH induced short luteal phase in anestrus ewes. J Reprod Fertil 1994;100:211-217.
13. Baltye HM, Fairclough RJ, Cameron AWN, et al: Evidence for prostaglandin involvement in early luteal regression of the superovulated nanny goat. J Reprod Fertil 1988;84:425-430.
14. Chimenineau P: Effects of a progestogen on buck induced short ovarian cycles in the Creole meat goat. Anim Reprod Sci 1985;9:87-94.
15. Zarkawi M, Al-Merestani MR, Wardeh MF: Induction of synchronized oestrous in indigenous Damascus goats outside the breeding season. Small Rumin Res 1999;33:193-197.
16. Leboeuf B, Forgerit Y, Bernelas D, et al: Efficacy of two types of vaginal sponges to control onset of oestrus, time of preovulatory LH peak and kidding rate in goats inseminated with variable number of spermatozoa. Theriogenology 2003;60:1371-1378.
17. Freitas VJF, Baril G, Saumande J: Estrus synchronization in dairy goats in use of fluorogestone acetate vaginal sponges or norgestomet ear implants. Anim Reprod Sci 1997;46:237-244.
18. Freitas VJF, Baril G, Bose M, et al: The influence of ovarian status response to estrus synchronization treatment in dairy goats during the breeding season. Theriogenology 1996;45:1561-1567.
19. BonDurant RH, Darian BJ, Munvo CJ, et al: Photoperiod induction of fertile oestrus and changes in LH and progesterone concentration in yearling dairy goats. J Reprod Fertil 1981;63:1-9.
20. Shelton M: Reproduction and breeding of goats. J Dairy Sci 1978;31:994-1010.
21. East NE, Rowe JD: Subcutaneous progestin implants verses intra vaginal sponges for dairy goats estrus synchronization during the transitional period. Theriogenology 1989;32:921-928.
22. Dawson LJ: Estrus synchronization in does. Proc Small Rumin Reprod. Kansas State University; 2011.
23. Pierson JT, Baldassarre H, Keefer CL, et al: Seasonal variation in preovulatory events associated with synchronization of estrus in dairy goats. Theriogenology 2001;56:759-769.
24. Chimeneau P, Normant E, Ravault JP, et al: Induction and persistence of pituitary and ovarian activity in the out-of-season lactating dairy goat after a treatment combining a skeleton photoperiod, melatonin and the male effect. J Reprod Fertil 1986; 78:497-454.
25. Amoah EA, Gelaye S, Guthrie P, et al: Breeding season and aspects of reproduction of female goats. J Animal Sci 1996;74:723-728.
26. Keisler DH: Endocrine control of reproduction in the ewe and ram. Proc Small Anim Rumin Short Course (ACT and SFT); 1994. p. 2-31.
27. Lincoln GA, Short RV: Seasonal breeding: nature's contraceptive. Recent Prog Horm Res 1980;36:1-43.
28. Karsch FJ: The hypothalamus and anterior pituitary gland. In: Austin CR, Short RV, editor. Reproduction in mammals: hormone control of reproduction. Cambridge: University Press; 1984. p. 1-20.
29. Goodman RL, Karsch FJ: The hypothalamic pulse generator: a key determinant of reproductive cycles in sheep. In: Follett BK, Follett DE, editors. Biological clocks and seasonal reproductive cycles. Bristol: John Wright Pub; 1981. p. 223-242.
30. Thiery JC, Pelletier J: Multiunit activity in the anterior median eminence and adjacent areas of the hypothalamus of the ewe in relation to LH secretion. Neuroendocrinology 1981;32:217-224.
31. Clarke IJ, Cummins JT: The temporal relationship between gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) recreation in ovariectomized ewes. Endocrinology 1982;111:1737-1739.
32. I'Anson H, Herbosa CG, Ebling FJ, et al: Hypothalamic versus pituitary stimulation of luteinizing hormone secretion in the prepubertal female lamb. Neuroendocrinology 1993;57:467-475.
33. Jansen HT, Khalid M, Jackson GL: N-Methyl-D,L-aspartate induces a transient increase in LH secretion in the seasonally anestrous ewe. Domest Anim Endocrinol 1991;8:55-62.
34. Kinder JE, Day ML, Kittok RJ: Endocrine regulation of puberty in cows and ewes. J Reprod Fertil (Suppl) 1987;34:167-186.
35. Karsch FJ, Goodman RL, Legon SJ: Feedback basis of seasonal breeding: test of an hypothesis. J Reprod Fertil 1980; 58:521-535.

36. Moss GE, Crowder ME, Nett TM: GnRH-receptor interaction. VI. Effect of progesterone and estradiol on hypophyseal concentrations of gondaotrophins in ovariectomized ewes. Biol Reprod 1981;25:938-944.
37. Reeves JJ, Arimura A, Scally AV: Changes in pituitary responsiveness to luteinizing-hormone, releasing hormone (LH-RH) in anestrous ewes pretreated with estradiol benzoate. Biol Reprod 1971;4:88-92.
38. Moenter SM, Caraty A, Lacatelli A, et al: Pattern of gonadotrophin-releasing hormone (GnRH) secretion leading up to ovulation in the ewe: existence of a preovulatory GnRH surge. Endocrinology 1991;129:1175-1182.
39. VonBrackel-Bodenhauser A, Wuttke W, Holtz W: Effects of photoperiod and slow-release preparations of bromocriptine and melatonin on reproductive and prolactin secretion in female goats. J Anim Sci 1994;72:955-962.
40. Wuliji T, Litherland A, Goetsch AL, et al: Evaluation of melatonin and bromocryptine administration in Spanish goats. Small Rumin Res 2003;49:41-49.

Pregnancy diagnosis and prepartum conditions affecting does

Lionel J. Dawson
Boren Veterinary Medical Teaching Hospital, Oklahoma State University, Stillwater, OK

Abstract

There are different methods utilized to diagnose pregnancy in does. Ultrasonography, hormone/pregnancy specific protein assays and radiography have emerged the most common methods used with a high degree of accuracy. Ballottement or abdominal palpation is performed when the doe is in her last trimester of pregnancy. Other methods described in literature, such as recto-abdominal palpation utilizing a rod (Hulet's rod), have been used in the 60's in the U.S., and still being used in other countries. Non-return to estrus 20-22 days after breeding is a good management practice utilized by producers to monitor estrus activity of the does during the breeding season. However, pathologic conditions of the uterus and ovaries, physiologic anestrus late in the breeding season and post-breeding anestrus in does bred out of season may prevent these does from returning to estrus after breeding. Choice of the method utilized in small ruminants depends on the availability of equipment, number of days after breeding, number of animals to examine, desired accuracy, need for quick results, cost to the client and experience of the examiner. A reliable technique for early detection of pregnancy would allow the producer to make decisions regarding early culling or rebreeding of barren does. Pregnancy diagnosis and estimating the length of gestation is very important in the treatment of certain prepartum conditions like pregnancy toxemia, vaginal prolapses and abdominal hernia to induce parturition in does if necessary.

Introduction

Gestation in a does is usually around five months (146-154 days). During the last decade there has been increasing awareness for the need for early pregnancy diagnosis in does. Examination of the goat for pregnancy may be done as a part of a reproductive health program or may simply be requested by the owner who would like to know the pregnancy status of his or her doe. During the last two decades, ultrasonography has been the most common diagnostic approach utilized in goats for early pregnancy diagnosis. Early applications of ultrasonography utilized amplitude or A-mode ultrasound.[1] A-mode ultrasound detects fluid filled structures but they are not pregnancy specific. They send sound waves from a hand-held transducer placed externally on the skin of the abdomen and directed toward the uterus. Sound waves are converted to electrical energy in the form of audible or visual signal. They detect fluid-filled organs at the depth of 10 to 20 cm in the abdomen. Some units will emit a light or audible signal when a fluid-filled structure is detected. Units with oscilloscopes will display reflections as peaks or blips on the screen. Accuracy for A-mode is around 80-85% if performed between 60 to 120 days during pregnancy.[1-3]

Doppler ultrasonography utilizes the principle of detecting movement such as bloodflow and heartbeat. For diagnosing pregnancy in a doe, the Doppler ultrasound detects bloodflow through the middle uterine artery, umbilical arteries, fetal heartbeat and fetal movements.[2-6] Hand held Doppler probes emit ultrasound waves. A wave reflected from motionless structures has the same frequency as the transmitted sound whereas sound reflected from a moving organ or blood has a different frequency. Differences in frequency are converted to an audible sound. Audible signals may be distinguished by the observer include the fetal heartbeat, arterial blood flow through the middle uterine artery and umbilical arteries, fetal movement and maternal intestinal movements. The transducer could be applied externally to the skin or intra-rectally using a rectal probe. Accuracy rate is higher when done in the third trimester.[2-6]

B-mode real-time ultrasonography is an accurate, rapid and simple imaging technique used for detection of pregnancy in goats. Trans-rectal or trans-abdominal ultrasonography applications can be used with nearly 100% accuracy rate.[7] Trans-rectal ultrasonography using an adapter with a 7.5 MHz or 5.0 MHz rectal probe can be performed as early as 25 days. It is advisable to perform routine rectal ultrasonography around 35 days after breeding to confirm pregnancy. If done earlier than 35 days, the diagnosis needs to be confirmed at a later date due to the possibility of early embryonic losses. Trans-

abdominal ultrasonography is usually done 40 days after breeding. Real time B-mode produces a two dimensional image on the screen. If the doe is pregnant a moving image of the uterus with fluid, fetus and placentomes may be seen. The fetal heartbeat is detected as early as 25 days trans-rectally and 35 days trans-abdominally. Placentomes are seen as early as day 40 and they appear as echogenic densities in the uterine wall. They are C-shaped by day 45, with the concave surface directed towards the uterine lumen.[7-24]

Hormones measured to aid in diagnosis of pregnancy are progesterone, estrone sulphate and pregnancy specific proteins.[23] Progesterone assays on the blood and milk are usually done 18-22 days after breeding. Progesterone tests more accurately detect non-pregnancy than pregnancy. They are not pregnancy specific; high levels could be seen in conditions such as hydrometra, early embryonic death, pyometra and fetal mummification. Estrone sulphate tests on blood and milk are done > 50 days after breeding. Errors may arise from improper handling of samples, improper timing of collection, unknown breeding dates and other factors. Pregnancy specific protein B (PSPB) and pregnancy associated glycoproteins (PAG) have been identified in various ruminant species including cattle, sheep and goats. Pregnancy associated glycoproteins are released by the trophoblast of the placenta after four weeks of gestatoin.[23] Pregnancy specific protein B and PAG tests are highly specific for pregnancy. Blood levels of these proteins may remain elevated for a short period after embryonic death or fetal death which could explain apparent false positive results. As with all biologic tests, false negative results are possible. Serum is used to detect these proteins and there are two laboratiroes offering this test: Bio Tracking and IDEXX laboratories. They are about 95% accurate.

Prepartum conditions or diseases

The most common pregnancy or gestational conditions affecting does are pregnancy toxemia, pseudopregnancy, vaginal prolapse, injury and abortions. Pregnancy diagnosis and estimating the length of gestation is very critical in the treatment of certain prepartum conditions such as pregnancy toxemia, vaginal prolapses and abdominal hernia when deciding to induce parturition as a treatment for these conditions.

Pregnancy toxemia (PT) is a metabolic disease or condition of does that occurs in late pregnancy, and may have significant economic effect. The primary cause of PT is a decline in feed intake during the last four to six weeks of gestation. During this period, fetal growth is very rapid and energy demands are increased; this is particularly true in does carrying multiple fetuses. Since the uterus, fetuses and placenta take up an increasing amount of abdominal space, there is less room for other organs to expand during late term pregnancy. If a doe is overly fat she also has less room to hold feed. Thus feed intake decreases and the doe is forced to break down fat stores for energy. Ketone levels in the urine and blood are elevated. While the body can use small amounts of ketones as an energy source, excessive amounts lead to development and clinical signs of PT.[25-29,32]

Pregnancy toxemia usually occurs in the last six weeks of pregnancy in goats, with peak incidence during the last two weeks of gestation. Older, especially thin or fat does carrying triplets or quadruplets are especially at risk although females carrying a single fetus or twins may also be affected. Environmental stress, management change, diseases, poor quality or quantity of feed offered can also contribute to the decrease in feed intake and facilitate the development of PT.[24,32]

Early signs of PT usually exhibited by separation from the rest of the herd, depressed appetite and reluctance to move due to painful swelling of the feet. Within three to four days, the signs progress to severe depression, teeth grinding, muscle tremors and neurologic signs such as head pressing, star-gazing and blindness. Affected animals are down, unable to rise. A "fruity" or "sweet" odor to the breath may be present. Without treatment, there is high mortality in does.[25]

Treatment of does that are still eating consists of drenching with two ounces of propylene glycol twice a day. Drenching with baking soda solution (15-20 gr in 50 mL water), Drench Mix with Energy Malt (Advanced Agri Solutions, Stevens, PA; 4 oz powder per quart of water), or oral calf electrolyte packets mixed in water may help correct fluid deficit, electrolytes and acid-base balance. High quality feed (alfalfa, concentrates) should be offered in small quantities at frequent intervals during the last month

of gestation. Vitamin B injections and transfaunation (transferring rumen contents from a healthy goat or cow) may also provide rumen microflora and help increase appetite. Does that stop eating or ruminating should be treated with intravenous fluids with dextrose and electrolytes. In PT cases that are not responsive to medical therapy removal of the fetuses should be considered as part of the treatment. Induction of parturition or emergency cesarian section will remove the negative energy drain on the doe caused by fetuses. Kids are more likely to survive if parturition is induced within five days of term (≈145 days of gestation).[25] Stage of gestation in does with unknown breeding dates may be estimated by measuring the bi-parietal and placentome diameter, monitoring fetal heartbeat, mammary secretions, and swelling and relaxation of the perineal area.

If a doe is very large, has a history of multiple births, and has been confirmed to carry multiple fetuses, an increase in energy intake during the last trimester of gestation may prevent negative energy balance. Avoid sudden changes in feed and provide high quality energy-dense rations and free access to mineral mixture. During late gestation does carrying multiple fetuses should be offered 3.5 to 5 pounds of high-quality hay such as alfalfa, and one to two pounds of grain; the latter fed gradually to prevent acidosis or grain overload. Body condition scores (BCS) is helpful to evaluate nutritional status and overall health of the flock. Body condition score recommendations for all production phases are as follows: maintenance 2-2.5/5; breeding 3/5; early gestation 3/5; late gestation 3-3.5/5; lambing/kidding 3.5/5; and weaning 2-2.5/5. Provide room to exercise and treat any diseases that may result in decreased feed intake.[25]

Vaginal prolapse is not very commonly seen in does. It is seen more in pygmy goats than in other breeds.[30] Vaginal prolapses are usually seen in does during the last trimester of gestation, does with body condition scores of >4, does which are in small pens with lack of exercise, and in does exposed to moldy feed high in estrogenic content. Hereditary aspects with laxity of the pelvic ligaments may also play a role. Treatment is similar to that in other species by reducing the prolapse and placing a retaining suture or device on the perianal area. Treatment by reduction is not very successful because of complications due to reduction which include an increase in incidence of dystocia and stillbirths.[31] Does with unknown breeding dates may be induced to kid after measuring the bi-parietal and placentome diameter, monitoring the fetal heart beat, mammary gland development, mammary secretions, and swelling and relaxation of the perineal area.

Injuries caused by fighting and head butting may cause weakening of the abdominal muscles leading to tears or rupture of the prepubic tendon and hernia of the viscera. The condition is mainly seen in older does which may have had three to four kid crops.[32] Ruptured prepubic tendon is best managed by inducing parturition close to term, followed by performing a cesarian section in 24 hours to save the kids.

Pseudopregnancy or hydrometra is seen in does with a history of being exposed to a buck/artificially inseminated, or in does which have never been exposed to breeding. The incidence is higher in does bred during the non-breeding season and transitional period. Affected does will have elevated progesterone, appear to look pregnant with weight gain and develop a mammary gland with milk. Some will go into labor and expel fluid which is called "cloud burst." If the condition is diagnosed early and treated these does will conceive subsequently, but the condition can reoccur. Treatment is mainly by regressing the corpus luteum with a luteolytic dose of prostaglandin.[33]

Abortions in does are caused by various factors including infectious, malnourishment, environment, stress, hormones and trauma. Late term abortions are usually due to stress or infectious. It is important to remember that many of the diseases causing abortion in goats are zoonotic and can be transmitted to humans. Gloves, protective clothing and boots should always be worn when collecting samples from the abortion and hands should be cleaned carefully after handling potentially infectious material. Pregnant women or immune compromised people should not assist with kidding or handling of aborted material. Always isolate the doe and dispose of all aborted material (fetus, placenta and fluids) by burning or burying them.

Chlamydophilosis is the most common cause of infectious abortion in goats and the causative agent is *Chlamydophila abortus*.[34] Clinical signs are late term abortions, a high number of stillbirths and weak kids. Naïve yearling does that have been recently introduced into an infected herd are usually the

animals that abort. *Chlamydophila* can also cause conjunctivitis (pink eye), and polyarthritis (arthritis in multiple joints), though the strains of the bacteria causing these diseases differ from those causing abortion. Organisms are usually shed in the feces. Does are exposed from direct contact with the aborted fetuses, placenta, infected vaginal discharge, or ingestion of contaminated feed.[34] After ingestion, the bacteria colonizes intestinal epithelium and spread systematically to the uterus. Infected bucks may transmit the infection through natural service.

A history of late term abortions, stillbirths and birth of weak kids is suggestive of chlamydophilosis. The aborted fetus may be fresh or decomposed in appearance. Female kids infected with the organism at birth may abort in their first pregnancy. Does exposed to this bacteria during the first half of gestation may abort in the last trimester of that pregnancy. Does exposed in the last half of gestation may abort in the subsequent pregnancy or have high incidence of stillbirths or weak kids. Once abortion has occurred, does appear to have immunity as affected animals seldom abort more than once due to chlamydophilosis. Although immune, they can shed the bacteria in the vaginal secretions when in heat, potentially infecting the other does that may be pregnant.[25,32,34]

Isolate aborting does from the herd for at least three weeks. Placentas and fetuses should be removed and burned or buried. To minimize exposure, ensure that all feed and water sources are protected from contamination. Treating all does in an abortion outbreak with tetracycline may reduce additional abortions by up to 50%. There is a vaccine approved for use in sheep in the US. The vaccine should be administered four weeks before breeding.[25,32]

Toxoplasma gondii is a protozoan parasite that can infect goats and is second in importance only to *Chlamydophila* as a major cause of infectious abortion in the mid-west. Cats are the primary or definitive host for toxoplasmosis, becoming infected by eating infected rats or mice. Most warm blooded animals (birds and mammals) are intermediate hosts. The parasite matures in the intestine of the cat and infective eggs or oocytes are passed in the feces which can infect goats and other animals if consumed. Other than cat feces, the only source of infection for does is by consuming the infected placenta or birth fluids from aborting does. Younger cats are more of a threat to spread the disease than are older cats. Less than 4% of persistently infected animals will transmit the parasite vertically through transplacental transmission. Cats develop immunity as they get older thus neutered adult males and adult females are less likely to be a source of infection.[32,34]

If does are infected early in gestation fetal death and resorption usually occur. Infection late in gestation results in mummification, stillbirths, and birth of weak neonates. Not all fetuses from the infected dam may demonstrate the organism. Aborted fetuses do not have significant lesions. Twin and triplet abortions often reveal fetuses in variable postmortem conditions – mummified to fresh. Diagnosis maybe based on the appearance of the placenta, small greyish white foci of necrosis, "rice gain" lesions typically found on the cotyledons. Another common finding is focal necrosis in the cerebral white matter in the brain of stillborn and weak kids.[34]

During gestation, all cats should be kept away from pregnant does. Remove all feed which may have been contaminated with cat feces and prevent cats from defecating in feeders, on hay bales, water troughs and bedding. There are no vaccines available in the U.S. for toxoplasmosis. Feeding decoquinate or monensin throughout pregnancy has been shown to have some protective effect and may reduce the incidence of abortion.

Q-fever is a bacterial infection (*Coxiella burnetti*) that causes fetal resorption, stillbirths, and late term abortions (5% to 35%).[34] Abortions tend to occur in naïve animals. Causative organisms are transmitted through the air and inhaled or are consumed via infected aborted material, feces, urine, milk, or grazing contaminated pastures. Tick bites may also be a source of transmission. *Coxiella burnetti* remains viable in the phagosomes of free living amoebae. The ability of this organism to survive in protozoa, along with the organism's resistance to dessication may play a role in maintaining the organism in the environment. Q-fever's primary significance is its zoonotic potential. Q-fever infects a wide range of hosts including cattle, goats, sheep, pig, cats, dogs, and wildlife. Some does will be carriers of the disease without showing any signs. Carrier animals shed the organism in milk, feces and uterine fluid at the time of parturition. Intense agriculture practices that place large numbers of naïve animals in small

areas can result in exposure and reinfection of pregnant animals. Signs include stillbirths and late term abortion. Aborted fetuses are often fresh with little evidence of autolysis. The placenta is often the only tissue affected and is extremely useful in confirming a diagnosis. Cotyledons are often diffusely thickened and multiple areas thickened, leathery, covered with greyish/white to brownish/red exudate. Some aborted goats will have a retained placenta.[34] There has been conflicting information on whether treatment of pregnant does during a Q fever abortion storm has an effect on the course of the disease. Manure should be composted for at least five months and spread only on still days. The organism is resistant to drying which means it aerosolizes and can be inhaled. This is a zoonotic disease meaning it can be contracted by humans so a mask should be worn when scraping manure or sweeping the area. Colostrum and milk have high levels or organisms so all milk should be pasteurized before drinking. There is currently no effective vaccine available.

Brucellosis can cause abortions in does and orchitis in bucks. While brucellosis in goats is usually caused by *Brucella melitensis*, they can also become infected with *Brucella abortus*. Historically, the number of *Brucella melitensis* abortions has been extremely low in North America, but more recently, sporadic outbreaks have been reported in goats in Texas and Colorado. *Brucella abortus* is rare in the United States, but can cause late term abortions, stillbirths and weak kids. Does may develop systematic illness and show fever, depression, diarrhea, lameness, mastitis, and weight loss.[34] There is no effective treatment and infected animals should be slaughtered. Wear protective gloves, clothing, and boots when assisting with birthing problems or abortions. Any brucellosis cases must be reported to state veterinarians. The disease is spread to humans by direct contact or by drinking unpasteurized milk or consuming products made from infected milk.

Camylobacter fetus subsp. *fetus, Camplobacter jejuni* subsp. *jejuni,* **and** *Campylobacter lari* can infect goats. *Campylobacter* (vibriosis) can cause late-term abortions; however they are rare in goats. The organism colonizes the intestinal tract of the adult animal usually without showing any signs of diarrhea. A bacteremia may occur in susceptible pregnant animals leading to infection of the uterus, fetal septicemia prior to abortions. *Campylobacter* is transmitted via ingestion of feces, vaginal discharge, aborted fetus and placenta of infected does. A common sign is a bloody, pus-like vaginal discharge before or after abortion. Cotyledons are enlarged, yellowish, and covered with a brownish/red suppurative exudate. Intercotyledonary areas are often edematous and hyperemic and usually lack any exudate. Variable amounts of serosangunious fluid with fibrin are present in both the thoracic and peritoneal cavities. The liver may show multiple multifocal areas of necrosis. Diagnosis is by culture of the internal organs. There is a vaccine available labeled for sheep.[34]

Other bacteria such as Leptospira, Listeria, and Salmonella can cause abortions. *Leptospira hardjo, Letospira pomona, Leptospira castellonis and Leptospira icterohaemorhagiae* have caused abortions in goats. Leptospira is usually a subclinical infection in goats but during the bacteremic phase they may show fever and develop renal disease. Leptospira are usually transmitted by the urine of infected does. Producers should ensure that feed and water sources are not contaminated with feces or urine and control rodents and other animals that may be a source for these diseases.

Listeriosis caused by *Listeria monocytogenes* can cause mid-to late-term abortions. The organisms are mainly spread by ingestion and inhalation. Metritis and septicemia are seen does after abortions. Salmonellosis can cause mid to late term abortions. The disease can cause systemic signs and uterine infection after abortions. Does become infected following ingestion of the bacteria which are shed in the feces of various animals including cattle, birds, dogs, cats, rodents, and some wildlife.

Viruses can also cause abortions in goats. Viruses causing abortions in does are BVD, caprine herpes, bluetongue and Cache Valley virus. See the table for the clinical signs, diagnosis, prevention and control.[34]

Summary

Pregnancy diagnosis and estimating the length of gestation is very critical in the treatment of certain prepartum conditions including pregnancy toxemia, vaginal prolapse and abdominal hernia when considering induction of parturition as treatment for these conditions. Measuring the diameter of the

placentome and biparietal diameter, with other signs such as mammary development, mammary secretions and fetal heartbeat could be used to make a decision to either perform cesarian section or induce parturition in the does with pregnancy toxemia. Late term abortions are usually due to stress or are infectious in nature. It is important to remember that many of the diseases causing abortion in goats are zoonotic and can be transmitted to humans. Good management of pregnant does with adequate housing, good nutrition, strict biosecurity, vaccination and prevention of undue stress could prevent late term abortions in does.

Table. Summary of infectious abortions in goats[25,27,30,34]

Disease	Transmission	Clinical Features	Diagnosis	Diagnostic Aids	Control
1. **Enzootic Abortion** (EAE, Chlamydial or Chlamydophila abortion): Caused by *Chlamydophila abortus* that affects sheep, goats, occasionally cattle and humans. They cause late term abortions. Abortion strains differ antigenically from strains producing polyarthritis (sheep and cattle) and conjunctivitis in sheep and goats (pinkeye).	Transmission is mainly by ingesting contaminated feed, water and the environment with vaginal secretions, placenta and aborted fetuses. Spread is more rapid when does are confined. Many carriers are seen in endemic herds. Infection at birth in kids kept as replacement does may be carriers through to their first pregnancy.	Late term abortions, stillbirths and birth of weak infected progeny are the most common clinical signs seen. Fetal mummification is occasionally seen. Female fetuses exposed in utero may abort during their first pregnancy; does infected in the last month of pregnancy may not abort until the next gestation period. Does seldom abort more than once.	A chorionitis with chlorionic epithelial cells packed with elementary bodies appears to be the essential lesion. Cotyledons are pale, greyish white and are necrotic with a dark brown exudate. Intercotyledonary areas are necrotic, thickened, opaque and leathery.	Impression smears of the cotyledon, placenta and vaginal discharge (but not fetal stomach) stained by the modified Ziehl-Nielsen or Gimenez stain. Organisms can be cultured in yolk sac of embryonating chicken eggs. PCR techniques on placental trophoblasts, spleen and liver are useful. Serology on the dam is unrewarding. But detecting antibodies in the fetal fluids is also useful.	Vaccine available; must be given to males and females 4-6 weeks prior to breeding, or use 150 mg of tetracyclines per head per day in the feed for 2-3 weeks prior to breeding: may continue this in their feed through the first half of gestation. Controlling abortion outbreak with tetracyclines (limited success): (1) 400 mg/head/day in feed or water for the last 60 days of gestation. (2) Use slow-release tetracycline (LA 200) 20 mg/kg injectable to start during the last 60 days of gestation. Owing to their long incubation period, once-a-week protocol may be adequate to decrease losses and is much less expensive. (3) Treat weak newborns with tetracycline. (4) Isolate aborting females and also those with weak born kids. Prevention: 1) LA oxytetracycline, 90 days and 120 days of gestation is very effective in preventing abortions. Onset of therapy after the start of abortions will only reduce abortion rate. 2) OTC or Aureomycin 4 gm Crumbles at the rate of 1 lb/8 ewes (500 mg/hd/day).

2. **Toxoplasmosis:** *Toxoplasma gondii* affects a wide range of animals as well as man. It is widespread and has been reported in Australia, New Zealand, Britain, Turkey, USSR, and North America. Cats and other Felidae are considered the primary host and excrete oocysts; species such as goats and man are regarded as secondary hosts. In these species, the organism is found in two forms: tachyzoites, which are actively multiplying and invasive, and found in the acute state of the disease, and cysts containing bradyzoites found in the chronic phase of the disease.	Oocysts excreted in cat feces are thought to provide the major source of infection. Congenital transmission from does to kids is also established. Further epidemiologic knowledge is required to establish how the disease spreads during an epidemic.	Does infected in the earlier stages of pregnancy either resorb the embryo, or fetal death and undergo mummification (often only one of a twin pair) may occur. Twin or triplet abortions, have variation in fetal ages – mummification to fresh fetus. Infection in late pregnancy leads to abortion and perinatal losses of kids. Many congenitally affected kids survive. Disease in the adult is generally asymptomatic, occasionally CNS signs develop. In endemic areas only younger does usually are affected, and may show the above clinical signs.	Placental changes may be the only gross lesions observed. Gross lesions of the cotyledons (numerous grey-white foci 1 to 3 mm in diameter) are indicative of the disease. Not all cotyledons are equally affected, and such lesions should be differentiated from nonspecific calcification. Focal leukoencephalomalacia in the CNS of stillborn kids, or kids dying shortly after birth is a common finding.	Histology of the cotyledon to demonstrate local areas of necrosis, mineralization and the organisms. Histology of fetal brain to demonstrate foci of glial cells and leukoencephalomalacia. Microscopy of the brain and cotyledonary villi sections to see tachyzoites and immuno histochemistry for antibodies is necessary to confirm your diagnosis. Precolostral serology of the kids is useful, but maternal serology is unrewarding.	Prevent exposure to barn cats. Don't allow cats to consume aborted fetuses in a toxoplasma abortion. Treatment: Decoquinate 2 mg/kg/day Or Monensin 15-30 mg/kg/head/day throughout gestation

3. **Salmonellosis** (Paratyphoid abortion): *Salmonella abortus ovis*, *S. typhimurium* and *S. dublin* have been associated with abortion in does.	Ingestion of contaminated food and water; usually shed from carrier animals. Does in later pregnancy appear more susceptible? Overcrowding and other forms of stress favor an outbreak. Unless the infecting dose is large or the strain exceptionally virulent, infection seldom causes clinical disease in the absence of some other predisposing factors resulting in stress.	Abortions, stillbirths, births of weak infected progeny that usually die within 7 days of birth. Does may show high fever before aborting; most recover, but some die from metritis and/or septicemia. Some does and newborn kids show diarrhea; in the kids this is usually fatal. Kids up to two weeks of age may show bronchopneumonia. When infection is endemic, abortions tend to be confined to the younger does.	No specific placental lesions seen. Swollen, pale hemorrhagic cotyledons with necrosis. Aborted fetuses show usual signs of intrauterine death. Septicemia lesions may be seen in those kids dying during or shortly after birth.	Culture of organisms from fetus, placenta and uterine discharge.	Antibiotic treatment on flock basis is not effective and is very expensive. Avoid overcrowding or stressing of does. Do not feed on the ground unless a new area can be used each day. For valuable individuals, supportive therapy (fluids) and antibiotics are recommended.
4. **Brucellosis:** *Brucella melitensis* affects goats and sheep and other species including man. It is seen in Europe, Mediterranean countries, Africa, Central America and rarely in the United States. *B. abortus* occasionally affects does. *B.ovis* affects rams – epididymitis, can cause infertility, early and late term abortions, still births and weak kids.	Ingestion is the main method of transmission, especially during the kidding period. Droplet inhalation and entry both through the conjunctival membrane and broken skin occasionally occurs. Venereal transmission following natural mating is rare.	Abortions in late pregnancy, stillbirths and birth of weak infected kids may occur. Congenital infections may persist throughout life (especially *B. melitensis*). Systemic effects may be seen in the dam with fever, lameness (associated with joint swellings), sometimes central nervous system (CNS) signs.	The essential lesion is placentitis, with edema and necrosis of cotyledons. The intercotyledons membrane may be thickened, yellow-brown necrotic areas, often with adjacent hemorrhage. Mucopurulent material may be adherent to the allantochorion. Fetus shows usual signs of intrauterine death.	Culture and direct microscopy are used to identify organisms that are plentiful in the placenta, fetal stomach and vaginal discharge of the doe. Modified Ziehl-Nielsen technique is satisfactory for staining for direct microscopy. Complement fixation ELISA, CF and PCR are available on sera of aborting does.	Test and slaughter policy can be used when the disease is prevalent. Testing of replacement animals. General hygiene at kidding.

5. **Listeriosis** (*L. monocytogenes* or *L. ivanovii*)	Mainly ingestion.	Abortion, stillbirths and weak kids Autolysed fetuses seen. Abortion occurs from day >50 of gestation. Some born alive but die. Metritis and septicemia common in females. Placentitis, around the cotyledon and intercotyledon areas. Kids grafted to the aborting females can contract listeriosis through the milk, develop septicemia and die.	Necrotic, greyish white foci (1 or 2 mm diameter) is seen in in the liver, spleen, kidneys, lungs, heart and adrenals; leathery placenta.	Culture from fetal stomach, liver and placenta. Fluorescent antibody test on the placenta.	Isolation of aborting females. Do not feed spoiled silage or poorly fermented silage. During outbreak administration of long acting tetracycline at 20 mg/kg every 72 hours. Chlortetracycline in the feed 300 mg/head/day.
6. **Leptospirosis** (*L. icterohaemorrhagiae, L. grippotyphosa, L. pomona, L. hardjo, L.canicola L.castellonis* and *L. bratislava* have been reported as primary causes of abortions in goats)	Secreted in the urine. Transmission is through skin or mucousal abrasions.	Clinical signs seen primarily in primiparous does. They include metritis after abortions, anorexia, anemia, jaundice, hemoglobinuria and death.	Fetal organs will be hemosiderin stained due to autolysis. Some edema of the intercotyledonary regions.	Dark field microscopy, Immunofluorescence testing and silver stains on placenta, fetal tissue and fluids. FA on the kidney. PCR on the urine. Paired serum samples from aborting does.	***Vaccine*** ***Rodent control*** ***clean water supply*** ***Isolation*** of aborting females. During outbreak administration of long acting tetracycline at 20 mg/kg every 72 hours. Chlortetracycline in the feed 300 to500 mg/herd/day during the outbreak.
7. **Q-Fever** (*Coxiella burnetii*): It affects sheep, goats, cattle and other wild life. This organism is shed heavily in placentas, birth fluid, colostrum and milk.	Inhaling dust, grazing contaminated pastures and tick bites. Infected does can shed in the feces after parturition.	Abortion primarily in the naïve animals. Late term abortions. Fresh fetuses. Some kids born alive. Aborting does usually retain their placenta.	Late term abortion and stillbirth. Placentitis with intercolyedonary areas thickened and leathery. Cotyledons are diffusely thickened with multiple areas of necrosis, covered with grayish/white to brownish/red exudate.	Serological testing is of little use. Paired serum samples may give a retrospective study of the flock. IFA is commonly used. ELISA along with IFA would strongly suggest coxiella infection.	Producers should burn or bury the placenta. Oral chlortetracycline 200 mg/head/day for 3 weeks. Long acting tetracycline 20 mg/kg given s/c or i/m every 3 days for 5 treatments.

8. **Caprine herpesvirus**	Direct – nasal and genital routes. Latent infection in adults and spread during stress.	Kids – viremia and enteritis. Ulceratic and necrotic lesions the entire GI tract. Adults – Vulvovaginitis, balanoposthitis, respiratory disease and abortions.	Clinical signs: Multifocal white necrosis in liver, spleen, kidney and lungs, mesenteric lymph nodes, thymus and liver.	BoHV-1 positive virus isolation on nasal and vaginal swab. PCR – blood and swabs. Intranuclear iclusion bodies in the placenta and internal organs of the aborted fetus.	Avoid stress. Buy animals from a clean herd. Avoid commingling with calves and sheep. BoHV-1 Infect sheep and goats but they are subclinical. CpHv-1 can infect sheep and calves and become latent. Reactivation has not been successful in sheep and calves.
9. **Border Disease** (hairy shaker disease). The cause is infection of the pregnant ewe and doe with a pestivirus closely related to, if not identical with bovine viral diarrhea (BVD) virus. The disease has been described in Britain, North America, New Zealand, Australia, Greece and Ireland. Several strains appear to be involved.	Vertical transmission from ewe to lamb during gestation is well established, and venereal spread of the disease seems likely. Surviving lambs can transmit the virus both vertically and laterally for years. Most of the more obvious clinical signs result from infection of pregnant ewes in the first half of gestation. Severe loss is likely if susceptible pregnant ewes are introduced to infected flocks or if infected ewes are mixed with resident ewes having no immunity to the disease.	A loss of potential progeny at any stage during pregnancy and in the postnatal period occurs. Infertility with a marked increase in barren ewes, fetal mummification and/or maceration, abortions, stillbirths and losses of lambs born alive are all features of the disease. When the fleece has developed, it tends to be hairy and may be pigmented. If born alive, lambs may show muscular tremors causing incoordination and difficulty in nursing.	Colyledons tend to be small for fetal age; they occasionally show areas of focal necrosis (1 to 3 mm). Fetal mummification; hairy pigmented coats if the wool has developed; fetus small for gestational age; muscular tremors and incoordination if lambs are born alive. When late gestation fetuses or young lambs encounter the disease, nodular periarteritis, which is slow to resolve, may occur.	CNS shows hypomyelinogenesis and the skin shows characteristic lesions on histologic studies. BVD-neutralizing antibodies in the serum of dam or lamb to virus isolation PCR.	Prevent commingling of pregnant does and ewes with cattle.

10. **Bovine viral diarrhea** A pestivirus has been implicated pigs, alpacas, sheep, goats and deer. Causes abortion in sheep and goats.	Commingling with cattle. Persistent infection of lambs, kids and calves born when mothers were infected during pregnancy.	Stillbirths;weak kids do not survive. Shaker kids with no changes in hair coat. Abortions at any stage. Skeletal defects on aborted fetus - arthrogyposis, anasarca and mummified fetuses. PI kids possible when a pregnant doe exposed to PI calf .	Necrotizing placenta	Virus isolation PCR Serology – ELISA or SN	Prevent commingling of pregnant does with cattle.
11. **Cache Valley virus and Akabane virus Cache Valley Virus** is common in the U.S.	Arthropod borne disease – mainly by mosquitoes and flies (Culicoides)	Infection in early pregnancy can result in wide range of deformities in the fetus, microencephaly, hydrocephalus, arthrogryposis and muscle atrophy. Joint malformation may cause dystocia. Late gestation can cause premature and still born kid.	Clinical signs Serology- precolostral serum or fetal serum for antibodies.	Serology on the doe. Virus isolation on the aborted kids may be difficult.	Fly and mosquito control.
12. **Bluetongue virus**	Culicoides	Goats are subclinical, infected ewes are febrile, swollen discolored tongue, muscousal ulceration, pulmonary edema, lameness and abortions. Infection early in gestation leads to fetal resorption. Affected late term may cause abortions, stillbirths, weak kids and kids with neural and ocular defects.	Clinical signs. Abortions - placenta is normal. Fetuses - lesions in the brain.	Serology – Sera from aborted fetuses and precolostral serum tested for antibodies to BTV. Virus isolation and PCR.	Fly control and vaccination.

References

1. Madel AJ: Detection of pregnancy in ewe lambs by A-mode ultrasound. Vet Rec1983;112:11-12.
2. Buckrell BC: Applications of ultrasonography in reproduction in sheep and goats. Theriogenology 1988;29:71-84.
3. Campbell EA, Herve M, Bell AT: Diagnosis of pregnancy in sheep using ultrasonic compound scanning. Aust Vet J 1969;45:40.
4. Fraser AF, Nagaratnam V, Callicot RB: The comprehensive use of Doppler ultrasound in farm animal reproduction Vet Rec 1971;88:202-205.
5. Fukui Y, Kimura T, Ono H: Multiple pregnancy diagnosis is sheep using an ultrasonic Doppler method. Vet Rec 1984;114:145.
6. Hulet CV: Pregnancy diagnosis in the ewe using an ultrasonic Doppler instrument. J Anim Sci 1969;28:44-47.
7. Haibel GK: Use of ultrasonography in reproductive management of sheep and goat herds. Vet Clin North Am Food Anim Pract 1990;6:597-613.
8. Campbell S: The prediction of fetal maturity by ultrasonic measurement of the biparietal diameter. J Obstet Gynaecol Br Commonw 1969;76:603-609.
9. Davy CG: An evaluation of pregnancy testing in sheep using a real-time ultrasound scanner. Aust Vet J 1986; 63:347-348.
10. Deas DW: Pregnancy diagnosis in the ewe by an ultrasonic rectal probe. Vet Rec 1977;101:113-115.
11. Dusik KT: On the possibility of using ultrasonic waves as a diagnostic aid. Neurol Psychiatr 1942;174:153.
12. Fowler DG, Wilkins JF: The identification of single and multiple bearing ewes by ultrasonic imaging. Proc Aust Soc Anim Prod; 1980. p. 492.
13. Fowler DG, Wilkins JF: The accuracy of ultrasonic imaging with real-time scanners in determining litter numbers in pregnant ewes. Proc Aust Soc Anim Prod;1982. p. 636.
14. Gearhart MA, Wingfield WE, Knight AP, et al: Real-time ultrasonography for determining pregnancy status and viable fetal numbers in ewes. Theriogenology 1988;30:323-337.
15. Haibel GK: Real-time ultrasonic fetal head measurement and gestational age in dairy goats. Theriogenology 1988;30:1053-1057.
16. Haibel GK, Perkins NR, Lidl GM: Breed differences in biparietal diameters of second trimester Toggenburg, Nubian, and Angora goat fetuses. Theriogenology 1989;32:827-834.
17. Haibel GK, Perkins NR: Real-time ultrasonic biparietal diameter of second trimester Suffolk and Finn sheep fetuses and prediction of gestational age. Theriogenology 1989;32:863-869.
18. Herring DS, Bjornton G: Physics, facts, and artifacts of diagnostic ultrasound. Vet Clin North Am Small Anim Pract 1985;15:1107-1122.
19. Hulet CV: Determining fetal numbers in pregnant ewes. J Amin Sci 1973;36:325-330.
20. White JR, Russel AFJ, Fowler DG: The use of real-time ultrasonic scanning in the diagnosis of pregnancy and the determination of fetal numbers in sheep. Vet Rec 1984;115:140-143.
21. White IR, Russel AFJ: Pregnancy diagnosis and fetal number determination. In: Marai IF, Owen JV, editors. New techniques in sheep production. London: Butterworths; 1987. p 207.
22. White IR, McKelvey WA, Bushby B, et al: Diagnosis of pregnancy and prediction of fetal age in red deer by real time ultrasonic scanning. Vet Rec 1989;124:395-397.
23. Bretzlaff KN, Romano JE: Advanced reproductive technique in goats. Vet Clin North Am Food Anim Prac 2001;17:421-434.
24. Dawson LT: Determination of fetal numbers in Alpine does by real-time ultrasonography. Sm Rumin Res 1994;14:225-231.
25. Dawson LT: Common diseases in goats. In: Meat goat hand book. Langston(OK):American Institute of Goat Research; 2007. p. 123-160.
26. Smith MV, Sherman DM: Nutrition and metabolic diseases. In: Goat medicine. 2nd ed. Ames(IA): Blackwell; 2007. p. 527-564.
27. Pugh DG, Baird AN: Pregnancy toxemia. In: Sheep and goat medicine. 2nd ed. St. Louis: Elsevier ; 2012. p. 200-201.
28. Bowen J: Feeding niacin may prevent disease. Dairy Goat J 1998;76:117.
29. Lalitha K, Rao DST, Suryanarayana C: Epizootiological studies on pregnancy toxemia in does. Indian Vet Med J 2001;25:391-392.
30. Dawson LJ: Kidding problem in does. Proc Caribbean Vet Med Assoc; 2012.
31. Braun W: Periparturient infections and structural abnormalities in does. In: Youngquist RS, Threlfall WR, editors. Current therapy in large animal theriogenology. 2nd ed. St. Louis: Saunders; 2007. p. 572-574.
32. Smith MC, Sherman DM: Digestive system in goats. In: Goat medicine.2nd ed. Ames(IA): Blackwell; 2007. p 390.
33. Braun W: Non-infectious infertility in a doe. In: Youngquist RS, Threlfall WR, editors. Current therapy in large animal theriogenology. 2nd ed. St. Louis: Saunders; 2007. p. 587-589.
34. Moeller RB: Disorders of sheep and goats. In: Njaa BL, editor. Kirkbride's diagnosis of abortion and neonatal loss in animals. 4th ed. Ames(IA): Wiley-Blackwell; 2012. p. 49-87.

Assisted reproductive technologies in small ruminants

J.E. Romano
Large Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX

Assisted reproductive technologies or techniques (ARTs) refer to achieving a pregnancy by artificial means or partial artificial means.[1,2] However, there is not complete consensus about this definition, because ART for others includes all the fertility treatments in which both female and male gametes are handled.[3] The first ART was artificial insemination (AI). Then the following ARTs were developed: embryo transfer (ET), in vitro production of embryos (IVP), assisted fertilization techniques (AFTs), gamete intrafallopian transfer (GIFT), zygote intrafallopian transfer (ZIFT), and cloning.[1,2] Most recent ARTs are the use of sexed semen, cytoplasmic transfer to zygote or oocytes, and others. Most of the ARTs are inter-related to each other; for example, the production of an embryo by in vitro fertilization will require embryo culture and further embryo transfer procedure to obtain an offspring.

Keywords: Artificial insemination, embryo transfer, intracytoplasmic sperm injection, cloning, assisted reproduction

Artificial insemination

Artificial insemination consists of the introduction of semen into the female genital tract with the help of some instrument.[4] It could be regarded as the oldest and most widely applied ART in livestock. Artificial insemination has changed the small ruminant industry and has fostered genetic improvement by using proven sires, storage of male genetic material,and use of this genetic material independent of time and space; it has also provided control of reproduction, allowed breeding of females out-of season, and restricted potential diseases that could be transmitted through the semen.[4-7] In general, AI is a simple, economical, and successful ART.

The equation of fertility for AI (also known as Bartlett's equation) is based on four components: estrus detection, estrus fertility, semen, and technician. The percentage of pregnancies resulting from AI is the product of the four factors and not the average of the four factors. The final results are multiplicative rather than additive.[8]

In small ruminants, estrus synchronization programs are used to concentrate estrus for either estrus detection or for AI at a fixed time. On the other hand, estrus synchronization protocols are not free of potential negative effects. In ewes, a substantial reduction in the total number of spermatozoa in the different organs (cervix, uterine horns, oviduct) of the female genital tract and times (samples collected at 1, 12, 24, 36, and 48 hours after AI with fresh semen) after estrus synchronization with intravaginal sponges compared with natural estrus was noticed.[9] The transport and survival of spermatozoa were affected by the progestogen-treated ewe.[9,10] The minimum number of fresh spermatozoa for natural or synchronized estrus is around 100 to 300 to 500 million, respectively.[10-12] In general, AI at a fixed time requires that the insemination be performed between 45 and 60 hours after pessary removal. However, the interval between intravaginal pessary removal and estrus, is affected by different factors and modifies the time to ovulation.[14,15] In small ruminants, a large variation in the time of ovulation after standard progestogen pessary treatment that has been shown to exist between some flocks may have resulted in less appropriate timing of insemination in some of the flocks/herds.[16-19] The factors could be responsible for the variation found among and within experiments using the same estrus synchronization protocols, the same batch of the same drugs, and the same personnel.[16-19] Among the factors that influenced this interval are: drugs used for estrus synchronization,[20-22,] presence of a male at the time of pessary removal,[23,24] presence of females around estrus at the time of pessary removal,[25] climatic conditions,[26] stress,[27] level of nutrition,[28] use of equine chorionic gonadotropin (eCG),[29] time of administration of eCG,[30] and dose of eCG.[31] In addition to these external factors, the potential influence of the stage of follicular ovarian activity could also be an internal factor that affect this interval.

The semen used for AI in small ruminants can be fresh, diluted and fresh, diluted and refrigerated, or frozen-thawed semen.[4]

In small ruminant AI, there are three basic possibilities for where the semen is placed: vaginal (VA-AI), cervical (CE-AI), and intrauterine through the cervix (transcervical AI, TR-AI) or through the abdomen (laparoscopic AI, LA-AI). The site of placement of semen has influence on pregnancy rates. There is a marked effect from vaginal, cervical, to uterine deposition on pregnancy rate.[32] The deeper the placement of the semen, the better the pregnancy rate.[33,34]

Sire effects on pregnancy rate have been described with the use of fresh semen,[11] with frozen semen after cervical insemination,[35-37] and after laparoscopic insemination.[17,38,39] In sheep, from several parameters studied on frozen-thawed semen, only the total and motile numbers of spermatozoa per inseminated ewe were associated with fertility ($r = 0.25$ and 0.26, respectively).[17]

In general, the fertility of refrigerated or frozen semen is lower than the fresh semen for the same quantity of spermatozoa per inseminating dose.[7,40] The storage of semen, either fresh, refrigerated, or frozen, causes ultrastructural, biochemical, and functional damage to the spermatozoa that results in reduction of motility, viability, and fertility compared with non-stored spermatozoa.[7] Different studies have revealed that preservation techniques induce sublethal damage that affects the functional capacity of spermatozoa; as a result, the spermatozoa behave differently from fresh spermatozoa even if they are fertile.[41,42] Irrespective of the type of extender/diluent or the technique of storage used, many spermatozoa show reduced motility and viability (longevity) and decreased fertility when maintained in refrigeration or frozen storage.[12,35,42-44] The low fertility rate of the frozen semen can be reverted by depositing the spermatozoa directly into the uterus.

Differences among semen sires in surviving the freezing and thawing process were detected. The reasons are unknown. Thus, knowledge of the ability of each sire is essential to determine the correct number of spermatozoa per each inseminating unit, as in the cattle, is required.[45] In general, wide variation in total number and motility after thawing among inseminating units has been found.[35,46] A difference from cattle, small ruminant's fertility is affected by multiple factors that interact with each other, making recommendations difficult. The number of spermatozoa per inseminating dose depends, among other factors, on: type of insemination procedure (vaginal, cervical, intrauterine trasncervical, or laparoscopic), type of semen (fresh, fresh diluted, refrigerated or frozen-thawed), time of the year (in-season or out-of-season), type of estrus (induced or natural), category of animal (pluriparous vs. nulliparous), and physiological state (lactating or dry).

Goat semen presents important differences compared with ram semen regarding the type of extender used for freezing semen. Goat semen has an inherent difficulty due to seminal plasma exposed to egg-yolk or milk-based extender that affects the viability of spermatozoa. Goat semen has an enzyme from the Cowper's glands (bulbourethral glands) which acts on lecithin of egg yolk producing lysolecithin that is toxic for the spermatozoa (egg yolk coagulating enzyme, EYCE.[47,48] In addition, seminal plasma contains a second enzyme that acts with some components of milk-based extender, depressing the survival of spermatozoa in vitro.[49] Different approaches have been used to deal with this situation. First, use extenders that do not contain any egg yolk.[50] Second, wash the semen to remove seminal plasma[51,52] and then use extender containing a high percentage of egg yolk. Third, use extenders containing egg yolk at no more than 2% at the final extender-semen dilution.[4,31]

Removal of seminal plasma by washing the spermatozoa (once or twice) immediately after semen collection increases the percentage of live cells, percentage of motile cells, motility rate, and percentage of normal acrosomes after thawing when extenders containing egg yolk, milk-based extender or TRIS are used.[51-54] The effect of washing was better for spermatozoa collected during the breeding season than for out-of-season collections.[30] On the other hand, elimination of the washing protocol is an important component to decrease spermatozoa loss and the mechanical damage to spermatozoa.[7,55] Different solutions have been used for washing the semen such as Krebs-Ringer phosphate buffer (with or without glucose), extenders without glycerol, and others.[51,52,54] The semen and washing solution ratios used were from 1:5 to 1:10, and centrifugation was for 10 to 15 minutes at 600 to 1000 g.[7,51,52]

The use of components of animal origin for extender preparation such as egg yolk, seminal plasma, bovine serum albumin, and milk proteins, increases concerns about the potential risk of microbial contamination by these products. Soybean lecithin (from 1 to 2%, weight/volume) is a real alternative as a chemically defined pathogen-free component to be part of the extenders instead of products of animal origin. Different studies have shown that these new extenders preserve the semen adequately after freezing and thawing compared with diluents containing products of animal origin.[50]

In small ruminants the method of dilution of semen for further freezing is based either on a constant rate (volume/volume) or constant spermatozoa concentration.[4,12] When the semen is diluted to a constant concentration, the quantity of glycerol/cell remains constant, compared with the constant rate (volume/volume ratio; 1:2 to 1:5), where the quantity of glycerol/cell is variable. Both approaches have produced successful pregnancies.[4,12]

In rams, length of storage in liquid nitrogen (LN) does not affect the fertilizing ability of frozen spermatozoa.[12,40] However, in bucks some studies found a decrease in percentage of motile spermatozoa during storage when skim milk-glucose extender[51] or tris-glucose-citric acid-yolk extender was used.[32]

In small ruminants, different reproductive parameters are used to evaluate the performance of a program. Estrus response is the number of females that showed estrus during a certain time from all the females that were submitted to treatment. The fertility rate is measured either as nonreturn rate, conception rate, pregnancy rate, or lambing/kidding rate.[56] The nonreturn rate is based in the number of females bred that do not return to estrus after exposure to a male; for example, nonreturn rate at 25 days. The conception rate is the number of pregnant females compared to the total number of females that were detected in estrus and bred. The pregnancy rate is the relation between the number of pregnant females and the total number of females in the breeding program. In the case of AI at a fixed time, the conception rate is identical to the pregnancy rate. The lambing/kidding rate is the relation between the number of females that lambed/kidded and the total number of females at the beginning of the breeding program. Fecundity is defined as the number of offspring produced and the total number of females that lambed/kidded. Prolificacy is defined as the relation between the total number of kids/lambs produced and the total number of females at initial at the breeding program.[19]

Vaginal insemination

Vaginal insemination is based on the deposition of an inseminating dose of semen in the anterior vagina without localization of the external cervical os.[4] This type of insemination requires the use of fresh or diluted semen containing a high number of spermatozoa for optimal pregnancy rate. In this insemination, there is a lot of semen loss due to backflow of spermatozoa.[43,44,57,58] It is not recommended with the use of frozen-thawed semen because the low fertility due to impaired transport of sperm through the ewe cervix when deposited in the vagina and/or entrance of cervix. The use of frozen-thawed semen by this route requires a high number of spermatozoa to obtain a satisfactory fertility rate.

Cervical insemination

Cervical insemination is accomplished when semen is deposited into the cervical canal.[4] There are different degrees of deposition, from shallow to deep.[33] In this technique careful observation of the external cervical os is required. If large amounts of mucus are present in the vaginal fornix drainage is mandatory. It has been reported in sheep and goats that deep deposition of semen into the cervix results in the highest pregnancy rate or lambing or kidding rate.[32,33] Cervical insemination could utilize fresh non-diluted semen, fresh diluted semen, or refrigerated diluted semen. The use of frozen-thawed semen containing high numbers of spermatozoa is only recommended for deep cervical insemination. In case of natural estrus or estrus synchronized with progestagen the minimum quantity of fresh spermatozoa per inseminating dose is not less than 100 and from 400 to 500 million, respectively.[12,13,59] The minimum number of frozen-thawed spermatozoa is between 600 to 700 million per female in estrus[12]or more.[34,59] Two cervical inseminations with frozen semen (1600 million of spermatozoa/dose) 12 hours apart during natural estrus resulted in a lambing rate of 50%,[60] however, this number of spermatozoa is more than 30 times higher than the dose used for intrauterine insemination by laparoscopy.[4]

Intrauterine transcervical insemination

In general, the goat cervix can be penetrated and the inseminating dose deposited into the uterus in around 25% to 60% of multiparous females.[31,32] Transcervical insemination is possible in does in either the standing or the over-the-rail position. In ewes, the cervix presents a series of anatomical characteristics that make penetration of AI pipette difficult due to its length, presence of multiple rings, caudally-oriented blunt annular folds, and rings positioned in an eccentric pattern.[61-65] In general, deposition of semen more than 10 to 15 mm into the cervical canal is not possible.[12,33] In ewes, the intrauterine insemination by the transcervical approach requires a special restraint system for the female and the cervix as well as special inseminating equipment.[66-69] Several factors influence the possibility of passing through the cervix in ewes; these include season,[46,70] category of animal,[71] parity,[33,46] interval from lambing to insemination,[46,70] breed,[71] estrus,[33,70] stage of estrous cycle,[65] and skill and experience of operator,[70,71] among other factors. The factors that increased the percentage of success of TR-AI were AI during the breeding season, AI in pluriparous ewes, AI within three to four months postpartum, AI while the animal was in estrus and when performed by experienced operators. New catheters for TR-AI are in development with promising results;[72] however, more research is required.

In sheep, the number of spermatozoa used by TR-AI were reported to be 80 million,[71] 150 million,[64,66,70] 200 million,[73,74] and 400 million.[75,76] In general, the numbers of spermatozoa used for TR-AI are higher than the recommended numbers used for LA-AI.[32,33]

The pregnancy rate from TR-AI using frozen-thawed semen was reported to be 19%,[71] 26%,[71] 29% ,[77] 47%,[74] 50%,[69] 64%,[76] 68%,[78] 70%,[74] and 71%,[73] from the total females in which intrauterine insemination was possible. These outcomes of TR-AI on pregnancy rate are similar that of LA-AI. However, the overall fertility rate for LA-AI was always superior to TR-AI due to the difficulties of placing the catheter inside the uterus in all females.

In sheep, varying degrees of damage were detected by histological examination on the cervical lining over the length of the cervix after the use of TR-AI needle.[77] In a previous independent study[79] of postmortem examinations of barren ewes, a high incidence of peri-cervical abscess and pyometra, possibly caused by the penetration of the reproductive tract were observed. All these side effects could affect temporary or permanently the further reproductive life of the ewe.

Laparoscopic insemination

Semen can be placed into the uterus by laparotomy or laparoscopy. The low fertility rate from frozen semen can be reversed by depositing spermatozoa directly into the uterus by laparotomy. However, a high incidence of pregnancy loss following this approach was detected.[57,60,80] However, the fertility was not affected when the same semen was deposited by laparoscopy. The latter method is quicker and less invasive and stressful for the inseminated females compared with laparotomy. The procedure needs to be performed in a clean, closed, and dust-free area. Laparoscopic artificial inaemination allows semen to be placed directly into the lumen of the uterine horns. The first report of this technique performed in ewes was by Killen and Caffery.[81] It is recommended that feed and water be withheld from the females at least 12 hours before the AI procedure. In sheep, this procedure could be performed with no sedation or with tranquilization or general anesthesia depending on clinician preferences as well as country, state, or other regulations. Due to inherent characteristics of goats, females require deep tranquilization to general anesthesia.[82,83] The female is placed in a laparoscopic cradle in horizontal position; the areas of puncture are sheared, and finally antisepsis is performed. Local anesthesia is applied to the selected area of trocar puncture. Later, the laparoscopic cradle is tilted head down to an angle between 40 and 60°. Pneumoperitoneum is carefully induced by introduction air or CO_2 through a Verres needle or intramammary catheter. Only a small volume of gas sufficient to separate the organs for easier observation and manipulation of the genital tract is required. Use CO_2 is preferred due to its high diffusion rate compared with air. Overinflation causes discomfort to the animal. The first trocar and cannula for the laparoscope (5 mm, 7 mm, or 10 mm diameter) is inserted into the abdominal cavity approximately 5 to 10 mm cranial to the udder and 5 mm to the left of the midline. The second

trocar and insemination cannula is inserted into the right side of the abdomen. The inseminating tools are either a special modified glass pipette or a special insemination gun designed for that purpose. The recommended site of puncture for semen deposition is the major curvature of the uterine horn. The needle of the inseminating gun or pipette is stabbed into the perimetrium, passing through the uterine wall to the uterine lumen. The same operation is repeated in the other uterine horn. One indication of the correct placement is the lack of resistance to expulsion of the semen and absence swelling at the uterine stab site. Then the instruments are removed, and excess CO_2 is expressed from the abdomen; only the skin is sutured or stapled and sprayed with an antiseptic. The animal is put in ventral recumbence and observed until complete recovery. Then, the female is allowed to return to covered yards or pens for several hours prior to being let out to pasture. Reduce any unnecessary stress for the next four weeks.

In sheep, a linear increase in pregnancy rate from 14%, 38%, 40.3%, to 73% was detected when the numbers of frozen-thawed motile spermatozoa per insemination were augmented from 1, 4, 16, to 64 million, respectively.[12,17,84] The same trend was reported by others.[32,33] A minimum of 20 million normal motile spermatozoa is necessary for LA-AI in fine-wooled breeds; however, the more seasonal breeds need approximately 40 to 50 million normal motile sperm to achieve acceptable fertility rates.[85] Lambing rates greater than 50% were only obtained when more than 20 million motile spermatozoa were deposited.[12,84]

In goats, 100 million total spermatozoa produce a kidding rate of 61% in animals synchronized with intravaginal pessaries and eCG.[86] In another study the same number of spermatozoa produced only a 24% rate of kidding.[87] In other reports, 200 million spermatozoa resulted in a pregnancy rate from 49% to 52%.[83,88] In Alpine goats 100 million spermatozoa are recommended and for Saanen goats 60 million spermatozoa are recommended for LA-AI.[7] In goats, LA-AI executed after estrus detection needs to be implemented between 12 and 24 after estrus detection when two estrus detections per day are performed (Romano, unpublished observations).

In sheep, LA-AI is also used in superovulated females to enhance the fertilization rate by using either fresh or frozen-thawed semen due to the high level of fertilization failure after natural mating.[84,89,90]

Embryo transfer

Embryo transfer is an ART by which at least one embryo is collected from a female (donor) and transferred to another female (recipient) that serves a surrogate mother for the remainder of gestation. The first successful embryo transfers in livestock species were performed on sheep and goats in Texas.[91,92]

The indications of embryo transfer are multiple, and include an increased number of offspring from a donor with valuable genotype for high quality productive traits, introduction of new breeds, importation and exportation of embryos, control of transmission of certain diseases, preservation of endangered species, breeding during out-of-season, required for other ART (i.e., in vitro production of embryos), for interspecific embryo transfer, as potential diagnosis for repeat breeders and research purposes. Embryo transfer requires strict selection of the donor female and recipients, an adequate superovulatory treatment, breeding, embryo collection and evaluation, potential embryo conservation, and finally transfer of the embryo.[93-96] Each of these steps is fundamental to obtain satisfactory results.

Multiple ovulation and embryo transfer (MOET) is one of the most frustrating ARTs in small ruminants, because the results vary from complete success to complete failure even when careful standard operating procedures are followed.[96,97] The main factors that are involved in the process are: variability in the superstimulation response, poor fertilization rate or failure, especially in females with high ovarian responses, surgical approaches for embryo collection, and early luteal regression.[94,96,97] These unpredictable results combined with high costs, difficulty of assessing the ovarian response, and use of surgical procedures for collecting and transferring the embryos, have prevented the use of MOET on a large scale.

The selection of the donor is the first step. The donor needs to be clinically healthy, free of internal and external parasites, have sound feet and legs, have good mothering ability, have adequate milk production, have previously lambed or kidded normally, and have a regular estrous cycle. Prior to

beginning the superovulation treatment, it is recommended that a reproductive examination including vaginoscopy and transrectal ultrasonography be performed. Also, collection of samples for testing for potential diseases is recommended, especially for export/import requirements.[98] For the recipients, most of the same criteria listed for donors are required.

Superovulation could be performed during the breeding season as well as the nonbreeding season. Better response is obtained during the breeding season. Several gonadotropins have been used for superovulation, including eCG, follicle stimulating hormone (FSH; porcine, ovine), equine pituitary extract (ePE), horse anterior pituitary hormone (HAPH), and human menopausal gonadotropin (hMG). The two most frequently used products for superovulation in small ruminants are FSH and eCG (also called pregnant mare serum gonadotropin, PMSG).[93,95,99,100] In general, the administration of FSH or eCG will start at the end of the luteal phase (day 12 in ewes and day 17 in does) or at the end of a progestagen treatment. Between 1000 and 2000 IU eCG is given at the time of pessary removal or one or two days prior to pessary removal as a single intramuscular injection due to its long half-life in vivo.[96,100] If FSH is used, several protocols can be used for superovulation; most commonly the injection of multiple doses of FSH from two to four days,or a single injection of FSH mixed with a long release carrier or a single injection of a combination of FSH and eCG.[96,101] In the multiple injection protocol, FSH was administered every 12 hours at a decreasing or constant dose.[95] In general, FSH was superior to eCG in number of corpora lutea, embryos recovered, and transferable embryos.[95,100,101] In addition, the proportion of nonovulatory follicles and short estrous cycle were significantly reduced with FSH compared to eCG.[95,100] To produce luteolysis, a single or second dose of prostaglandin F2α is used at the fifth and sixth FSH injection in the three-day protocol or at the time of eCG administration or 24 hours later. In general, donors will show standing estrus at approximately 24 hours after progestagen removal and earlier than synchronized recipients; therefore, recipients need to have their intravaginal pessaries removed 12 to 24 hours earlier than the donors. The benefits of eCG are a single dose and low cost; however, the long half-life produces persistent ovarian stimulation that could produce variable results and affect the number and quality of embryos collected. In addition, in goats, repeated eCG treatments are followed by decreased fertility in females inseminated at a fixed time after treatment due to the production of antibodies against eCG. However, this response was variable depending on the goat population.[102] The major histocompatibility complex (MHC) was involved in these immune responses to eCG.[103] To reduce this deleterious effect of eCG, antibodies against it were used in donor goats and ewes and the results showed this to be an efficacious treatment that increases the number of viable embryos collected compared with eCG alone.[104,105] A new alternative for superovulatory treatment in goats is the active immunization against inhibin which enhances ovarian follicular development and ovulation rate by promoting an increase in pituitary FSH secretion.[106] The superovulatory response to FSH is positively correlated to the number of small follicles of 1 to 2 mm and negatively correlated to the number of large follicles determined by laparoscopic observation prior to gonadotropin administration.[96]

The variable response to superovulation is the bottleneck of ET as it is in cattle.[93,96,97] Multiple factors influence the response to superovulation, such as individual and group sensitivity to gonadotropins (there are breed differences in response), breeding season, age of donor, level of stress, nutritional status, drug used for superovulation, and batch number, among others.

Donor females are bred by natural service, AI or a combination. In case of natural service, several controlled matings need to be performed per estrus. It is mandatory that donor males have a previous satisfactory breeding soundness examination. Hand mating is recommended every 6 to 12 hours throughout estrus. In donor ewes fertilization failure due to the effect of either the pessary or gonadotrophin treatment or both causes impaired spermatozoa migration. Therefore, to reduce the chances of this potential problem, fresh semen deposited by the intrauterine route (laparotomy or laparoscopy) is recommended. In donor does however, high fertilization rates occur after natural mating.[95] The frequency of fertilization failure was high in does treated with eCG compared with FSH. Fertilization failure also took place when a high superovulatory response occurred.[96]

Use of fresh or refrigerated semen requires cervical or transcervical depositions with high numbers of motile spermatozoa per insemination every 6 to 12 hours, with at least two inseminations per

estrus. Frozen-thawed semen is not recommended by the vaginal or cervical route. In sheep, frozen-thawed semen requires intrauterine deposition by laparoscopic or laparotomy insemination due to reduced viability and fertilizing capacity. However, intrauterine deposition of semen by laparotomy has been associated with a high level of embryo loss. In goats, it is possible to use intrauterine insemination by the transcervical approach; however, as mentioned previously, this is not achievable in all females. In does, fertilization failure is not a problem as in ewes after natural mating. Therefore, service every 12 hours is recommended. It is mandatory with the use of frozen-thawed semen that information about the percentage of normal spermatozoa and total and motile number of spermatozoa per dose be available.

One of the difficulties in small ruminants compared with large ruminants is the impossibility to assess the ovulatory response through palpation per rectum prior to embryo collection because most of the procedures for embryo collection are invasive. The use of laparoscopy or transrectal ultrasonography could be used to assess the number of corpora lutea to decide whether to proceed to embryo collection. These approaches are less invasive than laparotomy. The measurement of progesterone prior to the embryo collection at days six to eight is another possibility; however, this test requires time to determine the progesterone levels, and it is not precise enough to determine the total number of corpora lutea.

The collection of embryos can be performed by laparotomy, laparoscopy, or transcervical approach.[93,107,108] The embryo collection from the oviduct can be performed at early stages or from the uterine horns after day four. Oviductal collection is performed on days three or four (estrus = day zero) and is basically performed by laparotomy. These embryos contain eight to 16 blastomeres and are easily differentiated from unfertilized oocytes. Oviductal collection is used in valuable donors that could otherwise undergo premature luteal regression. However, this recovery requires oviductal transfer if no culture (in vitro or in vivo) is obtained. The side effect of this collection is that the high level of ovarian and oviductal adhesions will reduce the reproductive life of the donor.

In ewes, optimum outcomes measured as number of corpora lutea and numbers of embryos collected were obtained by laparotomy and laparoscopy. The best results of uterine collection are between days six to eight after estrus (estrus = day zero). In general, an embryo with an intact zona pellucida is a requirement to reduce the potential of transmission of certain diseases through these embryos by washing multiple times, especially for import or export conditions.[98]

Drawbacks of the surgical approach are stress to the animal, the requirement of general anesthesia and the formation of adhesions. Repeated laparotomies and handling of the reproductive tract can traumatize tissues and increase the incidence of abdominal adhesions, thereby reducing the potential of reproductive life of the donor female. After three procedures, there is a significant reduction of collection of embryos.[109,110]

Laparoscopy is less invasive; the incision wounds are smaller, and the incidence of infections is reduced. Additionally, there is less dehydration of tissue, less adhesion formation, and faster recovery than laparotomy.[111] However, laparoscopy requires special equipment and highly skilled and trained personnel. In ewes, repeated embryo collection was possible up to three times a month with an efficiency of approximately 60% to 80%.[112-114]

The first nonsurgical embryo collection from small ruminants was reported in goats.[115] Nonsurgical uterine collection seems a viable alternative to conventional surgical and laparoscopic procedures. In goats, pretreatment with prostaglandin E_2 and estradiol 24 hours prior to embryo collection was effective to permit passage through the cervix; however, no information about embryo recovery rate was presented.[116] In later research, laparoscopic collection had a significantly higher embryo recovery rate than transcervical collection (79% vs. 37%).[117] Trasncervical embryo collection was successful (90% recovery) when prostaglandin F2α was administered 16 or eight hours previor collection with oxytocin administered prior to flushing performed at day six.[118] In sheep, nonsurgical collection by the transcervical route was performed; however, the percentage of success was only 42%, and only 74% of the ova were recovered.[119] In ewes, transcervical passage was 100%, with a recovery of 65% in ewes pretreated with prostaglandin E_2 and estradiol cypionate 24 hours prior to collection.[120] In a further study, the transcervical technique of embryo collection was successful in around 46% of the multiparous ewes and in about 5% of nulliparous ewes submitted for hormonal treatment of prostaglandin

E_2 and estradiol cypionate the day before collection. Transcervical embryo collection was 60% and similar to laparoscopic collection.[121]

Several flushing media for embryo collection have been used. Basically, all contain a balanced salt solution with a source of protein and antibiotics. The source of protein can be either homologous serum or bovine serum albumin. If serum is used, it should be heat treated at 56°C for 30 minutes to inactivate the complement and it should be filtered through a 0.45 μm Millipore filter before use. The most used medium for embryo collection is Dulbecco's phosphate-buffered saline. Other media that can be used for flushing are Tissue Culture Medium-199 (TCM-199), Ham F-10 medium, and Brinster Mouse Ova Culture Medium-3. All these media are commercially available. TCM-199 with Hanks' salts do not depend on CO_2 for buffering, but this medium with Earle's salts, Ham-F10, and Brinster Mouse Ova Culture Medium-3 must be maintained under an atmosphere of 5% of CO_2 in air.[94,97,110]

The criteria of evaluation of small ruminant embryos are the same as those for cattle. The two criteria are stage of embryo development and cytological characteristics at embryo collection day. The embryo development needs to be in concordance with the day of embryo collection. Embryo flushing at day six is expected to collect compact late morula and early blastocyst. The collection of cleavage embryos at those days containing two to four cells suggests nonviable embryos, and presence of 16 to 32 cells means retarded development. Blastomeres should be reasonably symmetrical, uniform, and spherical, with a minimum of extruded cytoplasm. Considerable experience is required to classify embryos. The embryo can be graded using International Embryo Transfer Society criteria.[98] Good quality embryos can be used for freezing and/or immediate transfer. Low-grade embryos should be used only for immediate transfer.

Ovine and caprine embryos can be cultured for one or two days in bicarbonate- or phosphate-buffered media or be slowly cooled to 4°C for up to two to three days. This last approach may allow asynchronous recipients to be in the adequate day for the transfer with the temporarily arrested embryo. The embryos for transfer could be fresh, refrigerated, or frozen-thawed. Depending of the age of the embryos, oviductal or uterine transfer can be performed. Oviduct transfer requires in most cases a laparotomy approach. In general, more embryo transfers are performed with embryos of morula or blastocyst stages, therefore requiring uterine placement. The transfer of these embryos can be performed through laparotomy, laparoscopy, and the transcervical route. However, optimal pregnancy rates were obtained by laparotomy and laparoscopy. Embryo transfer by laparotomy involves some degree of trauma and often leads to the formation of adhesions that affect the uterus, oviducts and ovaries. This situation could temporally or permanently affect the future reproductive life of the female. The advantages reported for laparoscopy are quicker transfer and reduction of genital tract adhesions with rapid recovery of recipient females compared with laparotomy. There is also a combined laparoscopy and laparotomy approach in which the endoscope is used to localize the uterine horn that is ipsilateral to the corpus luteum and then raised to the level of the abdominal wall. Then the embryo is transferred via micropipette.[122] In ewes, laparoscopic transfer was performed through the paralumbar fossa in the standing position[123] or with general anesthesia in females in dorsal recumbency with and without exteriorization of the uterine horn.[124,125] The number of embryos transferred varies from one to three embryos per recipient. It is recommended that embryos be transferred to the terminal half of the uterine horn ipsilateral to the ovary containing the most developed corpus luteum or corpora lutea.[125]

Precise synchrony of estrus between donor and recipient is paramount. Asynchrony of plus or minus one day is acceptable; however, the best results are probably obtained when donor and recipient are at the precisely the same point in the estrous cycle.[126] In does 24 hours of asynchrony is well-tolerated compared with ewes, in which more than 12 hours of asynchrony is not well-tolerated based on pregnancy outcomes. Furthermore, the development stage of the individual embryo, rather than the day of estrous cycle of collection should decide which recipient day is used. For example, a compact morula should preferably be transferred to a recipient on day five or six rather than on day seven even though the donor was collected on that day.[127] The same principles are used for cultured embryos.

A good pregnancy rate after embryo transfer is between 55% and 65%.[110] However, this outcome depends on recipient condition, type of embryo transfer, kind of embryo, number of embryos transferred,

place of transfer, and other factors. In goats, the pregnancy rate was not different between laparoscopic embryo transfer and transcervical embryo transfer (36% vs 39%, respectively.[110,117]

The incidence of premature luteal regression in superstimulated goats is considerable and varies from 10% to 30% or higher. That has resulted in low embryo recovery rates with poor quality embryos.[99,100]

Short estrous cycles have also been reported at the beginning and end of the breeding season, after estrus synchronization with luteolytics, and after abortion.[95,99,128] Short estrous cycles occur between five and nine days after stimulated estrus. Short estrous cycles are due to anovulation and/or early luteal regression. The levels of peripheral progesterone during this period are low compared with does without early luteal regression. The recovery rate from short estrous cycles is reduced and the quality of embryos is low.[95] The observation of ovaries by laparoscopy during short estrous cycles around days five to nine is characterized by the presence of small white-pink corpora lutea. Different approaches have been used to deal with this situation: use of progestagen during the initial stage of metestrus and diestrus;[129] administration of antiluteolytic drugs during metestrus and early diestrus;[130] administration of luteotrophic hormones such as human chorionic gonadotrophin or gonadtripin releasing hormone during metestrus;[131] or collection of embryos at day three after breeding. In this last case, an oviduct flushing approach is required. In general, this estrus is estrus.

In vitro production of embryos

In general, this technology implies in vitro oocyte maturation (IVM), sperm capacitation, and in vitro fertilization (IVF) and in vitro culture (IVC) of early cleaving embryos until their development to morula or blastocyst stages (transferable embryos). The good quality transferable embryos can be cryopreserved. Some of these steps can be performed in vivo also and then moved to in vitro (oocyte can be from an ovulated follicle) and submitted to in vitro fertilization and vice versa.

In goats the first in vitro fertilization was obtained after capacitation of spermatozoa in a rabbit oviduct.[132] The first birth using IVP on ovulated oocytes was achieved by Hanada.[133] The first pregnancy from an oocyte matured and fertiiized in vitro was reported by Younis in 1991.[134] However, the first kid born from an oocyte matured and fertilized in vitro was informed two years later by Crozet.[135] Finally, in 1994, the first kid was born after all the steps were performed in vitro (IVM/IVF/IVC).[136]

In most studies in goats oocytes were recovered surgically by laparotomy,[137] by laparoscopy,[138] transvaginal ovum pick-up[139] or after the slaughter of animals that had been treated with or without hormones.[137,140] The oocytes were obtained from adult goats, prepubertal goats, and pregnant goats.[141] In goats the fertilization rate of in vitro matured oocytes was the same as those from recently ovulated oocytes.[137] When the entire ovary is available, the collection of oocytes can be performed by aspiration, puncturing, and slicing. The slicing method is simple, rapid and efficient compared with aspiration and puncturing.[134,142] In prepubertal goats, the number of ovarian follicles is lower than in adult animals, making it difficult to collect high numbers of oocytes compared with adults.[140] It is possible to obtain oocytes from pregnant females after hormonal treatment (eCG) with good results within the first four months of pregnancy.[141]

Oocytes with three or more layers of cumulus cells showed better capacity for in vitro maturation than oocytes with fewer layers of cumulus cells or no cumulus cells.[140] The development competence of goat oocytes was acquired progressively during follicular growth; oocytes collected from large antral follicles have the capacity to progress to the blastocyst stage, after in vitro maturation, fertilization, and culture.[135] Frozen-thawed oocytes were used, but fertilization was low.[143] Oocytes from small and medium-sized antral follicles yield a significantly lower proportion of blastocysts than those from large follicles.

Culture media used for IVM such as Tyrode albumin lactate pyruvic (TALP), modified Dulbecco's phosphate buffered saline, Tissue Culture Medium-199 (TCM-199) with 20% heat-inactivated goat serum with or without hormones have produced satisfactory results. The duration of in vitro maturation is approximately 24 hours. Most investigators maintained a droplet of medium under paraffin oil and in a humidified environment of 5% CO_2, 5% O_2, and 90% of N_2 at 38.5°C. The use of

FSH was effective at 10 μg/mL in the media.[144] It was found that the combination of TCM-199 supplemented with hormones (FSH, luteinizing hormone, and estradiol) supplemented with 10% fetal calf serum was the most efficacious medium for IVM and subsequent embryonic development.[145] The most frequent medium for in vitro maturation of oocytes is a culture medium supplemented with FSH, luteinizing hormone, estradiol, and 10% fetal calf serum. The extrusion of the first polar body at MII occurs in small ruminants between 16 and 24 hours after the beginning of maturation.[95]

In vitro fertilization was obtained satisfactorily by using fresh semen. The semen was prepared by using heparin in the medium from 2.5 to 10 μg/mL depending on the male.[146] The process requires an adequate level of calcium in the medium. Caffeine depresses the fertilization rate in goats.[147] The number of spermatozoa per oocyte used was from 400 to 100,000 to 200,000 spermatozoa.[137,145] The duration of IVF was also variable, from six to 24 hours.[136,148]

Studies of IVF have shown that oocytes and sperm can unite in a different oviduct. For example, when oocytes and spermatozoa from goats are placed in the sheep oviduct, fertilization will occur (gamete intrafallopian tube transfer). The inverse also is possible, oocytes and spermatozoa from sheep in a goat oviduct. The fertilized oocyte can be cultured in vivo or in vitro. In vivo culture was performed in oviducts of the same or other speciest (xenoculture). The in vitro co-culturing of two- or four-cell embryos to obtain morulas or blastocysts was successful using oviduct cells rather that uterine cells or without co-culture.[149] Fertilized goat oocytes placed into the oviducts of pseudopregnant rabbits developed to the morula or blastocyst stage.[150,151] Synthetic oviduct medium supplemented with serum proteins and amino acids under specific levels of 0_2, CO_2, and N_2 resulted in high levels of transferable embryos.[152,153]

The cleavage embryo can be cultured in a medium without or with cells (co-cultured medium). Multiple studies have shown that there is an in vitro block at the stage of eight to 16 cells that can be overcome by using co-cultured cells with supplemented serum.[154] More transferable embryos are obtained in co-culture systems.[152] Different types of cells were used in the co-culture media such as caprine cumulus cells, bovine cumulus cells, oviduct epithelial cells, bovine oviduct cells and porcine uterine epithelial cells. Caprine cumulus cells produced a high proportion of morula forms; however, the caprine oviduct epithelial cells produce more blastocysts. Therefore, a co-culture that involves a sequence of two co-cultures using first caprine cumulus cells and then caprine oviduct cells seems to be appropriate.[136]

In small ruminants, transfer of in vitro-produced embryos resulted in lower pregnancy rates compared with in vivo-produced embryos.[153,155] This is probably because of differences in cell structure, biochemistry and other differences that can make these in vitro-produced embryos more sensitive to the freezing/thawing process.[156]

Intracytoplasmic sperm injection

Intracytoplasmic sperm injection (ICSI) is the ART procedure that consists of the microinjection of single spermatozoa across the plasma membrane and inside of the cytoplasm of a metaphase II oocyte leading to fertilization. This method bypasses the process of spermatozoa selection and interaction with the oocyte; it could also use immotile or dead spermatozoa.[1,2] Intracytoplasmic sperm injection was reported for the first time in hamsters.[157] In small ruminants, this technology produced a lamb for the first time in ewes in 1996;[158] it was later reported in goats in 1997.[159] However, the first goat born by ICSI was described some years later in 2003.[160]

The indications for ICSI are: previous failure of IVF, depression of semen parameters (low concentration, low motility, high levels of abnormal spermatozoa, and low total number of spermatozoa), frozen semen (low number of spermatozoa, low motility), few oocytes from in vivo- or in vitro-matured process for fertilization (also cryopreserved).[1,2] In small ruminants, ICSI has potentially important applications in animal production systems, primarily its use with semen of valued animals, with testicular sperm, with epididymal sperm, oocytes from prepubertal females, cryopreserved oocytes, or other possibilities.[161] Intracytoplasmic sperm injection was shown to be an effective technique to solve a problem when few straws of frozen semen from a ram with altered genes for hemophilia A were

available. The use of a small injection pipette was effective in producing a number of blastocysts that were transferred and produced numerous live offspring, of which some had the desired genotype.[162,163] The use of sperm-mediated gene transfer and its combination with ICSI has the potential to be an interesting approach as a transgenic technique for transgenic animals.[161]

Cloning

The first approach to produce cloned embryos was by embryo splitting.[164,165] Embryo splitting produces monozygotic twins. A four- to seven-day-old embryo is surgically dissected with a glass knife or steel blade into halves or embryo parts. The demi-embryo is placed into the original or surrogate empty zona pellucida. This demi-embryo can be immediately transferred or can be cultured for one or two days to evaluate the development rate before transfer. Best results were obtained when two demi-embryos were transferred per recipient.[166]

Another method is separating the totipotent blastomeres from the inner cell mass from early embryos and putting individual cells into a surrogate empty zona pellucida. This possibility will create more identical animals than the preceding procedure. Willadsen[127] produced identical twins by microsurgical separation of the blastomeres of two-cell embryos, their insertion into a foreign zona pellucida, embedding them in a protective cylinder of agar and cultured in a ligated sheep oviduct. In a further study, blastomeres from four-cell embryos or eight-cell embryos were used, then cultured to develop to blastocyst stage and further transferred to recipient females producing pregnancies[167]

The third procedure for cloning consists of enucleation of oocytes in metaphase II (as a source of cytoplasm, named cytoplast), insertion of the donor cells (or nuclei, as a source of the nucleus, named karyoplast), activation of the reconstructed embryo, and in vitro culture. At the beginning of this new technique of cloning, the donor cells used were the totipotent blastomeres originated from the inner cell mass and transferred to an enucleated mature oocyte to produce a new embryo.[168] These findings indicated that at least some nuclei derived from transcriptionally active embryos are totipotent and able to be reprogrammed to support full-term development when fused to enucleated secondary oocytes.[169] This new embryo can be used for immediate embryo transfer or a source of more blastomeres. A further improvement of the type of cloning technique is to use embryonic cells rather than adult differentiated cells. This last procedure is named somatic cell nuclear transfer (SCNT). The first mammal cloned by using differentiated mature cells obtained from the mammary gland from a ewe was Dolly.[170] Then two years later the first goat produced by this type of cloning was reported.[171] This tactic destroyed an old myth in biology that a differentiation in cells cannot de-differentiate anymore and in addition opened a new concept in mammal reproduction, asexual reproduction.

The benefits of cloning by somatic nuclear transfer can be seen in superior genetic animals to increase their numbers (reproductive cloning) or in animals that cannot reproduce naturally or by using one of the other ARTs (ovarian or uterine pathology, orchiectomized or ovariectomized animals, terminal animals, or recently deceased animals).[165,172] In addition, this technology is a powerful resource for understanding the cellular and molecular aspects of nuclear re-programming.[172]

In general, a sterile skin biopsy from the ear containing fibroblasts (as the source of karyoplast) is obtained, then cultured in the laboratory, and finally used immediately for cloning or preserved in liquid nitrogen for further cloning. The source of the cytoplast could be in vitro or in vivo mature enucleated oocytes in metaphase II. Different cloning approaches for inserting the donor cells are used. The fibroblast is included in the perivitelline space of the cytoplast and then activated and electrically fused; only the nucleus of the fibroblast is injected directly into the cytoplast. Subsequently, the cloned embryos are cultured in vitro for a period, and when they reach the optimal stage for embryo transfer, they are transplanted into a surrogate mother animal. In general, the efficiency of this process is very low.[172,173] There is a lot of loss, not only during the in vitro process, but also during in vivo process (high level of pregnancy loss). Different types of cells have been used a source of karyoplasts such as fibroblasts, granulosa cells, Leydig cells, and others. A recent investigation opened a new dimension of potential cells such as somatic cells found in frozen-thawed semen, which can be used as nucleus donors to produce cloned blastocyst-stage embryos[174]

Production of transgenic animals

Transgenic animals are expected to have high impact in the future genetic improvement of livestock).[97] However, the drawbacks of this approach are low efficiency of the technique and high costs involved. There are growing numbers of recombinant proteins that have been expressed in milk. Commercial production of human pharmaceutical proteins in the milk of dairy goats has already been achieved. Transgenic goats carry the genes for a longer-acting form of tissue plasminogen activator (tPA); some goats are capable of producing this factor at level of 2 g/L of milk.[175] Transgenic cloned goats were produced using fibroblasts transfected with human coagulation factor IX as a source of karyoplasts.[176] Also, transgenic goats for human acid beta-glucosidase have been produced.[177] Additionally, removing immunogenic factors that are expressed in milk could be an interesting way to control epidemic diseases through drinking milk. Therefore, this new concept of transgenic animals as bioreactors opens a new dimension for pharmaceuticals products. Efficient transgenic animal production provides several new opportunities for agriculture and medicine.[172]

Other technologies

It is possible to pre-select the sex of offspring from different species prior to fertilization with an accuracy of 85-95%.[178] In small ruminants, sexed semen has been used for AI in estrus synchronized females,[179] superovulated females,[180] in vitro fertilization,[181] and the possibility of used in ICSI.[161] In sheep, the results are satisfactory compared with the results in cattle.[179,180,182]

Other new technology available is the use of ooplasmic (cytoplasm) transfer to an ovulated oocyte or zygote.[183] This new technology could be used in certain cases of mitochondrial disorders.

References

1. Meniru GI: Cambridge guide to infertility management and assisted reproduction. Cambridge: Cambridge University Press; 2001. p. 276.
2. De Jonge CJ, Barrat CLR: Assisted reproductive technology: accomplishments and new horizons. Cambridge: Cambridge University Press; 2002. p. 431.
3. Centers for Disease Control and Prevention: Assisted reproductive technology. Available at: http://www.cdc.gov/ART. Accessed May 10, 2013.
4. Evans G, Maxwell WMC: Salamon's artificial insemination of sheep and goats. Cape Town: Butterworths Pty Limited; 1987. p. 194.
5. F.A.O.: Disease control in semen and embryos. FAO animal production and health paper n 23. Rome: Food and Agriculture Organization of the United Nations; 1981. p. 101.
6. Garner DL: Artificial insemination. In: Cupps PT, editor. Reproduction in domestic animals. 4th edition. Waltham(MA): Academic Press;1991. p. 251-278.
7. Leboeuf B, Restall B, Salamon S: Production and storage of goat semen for artificial insemination. Anim Reprod Sci 2000;62:113-141.
8. Pace M: Has the fertilizing capacity of bovine spermatozoa changed? In: Reproductive loss in dairy cow: is the trend reversible? W.E. Petersen Symposium. St. Paul: University of Minnesota; 2003. p. 13-21.
9. Quinlivan TD, Robinson TJ: Numbers of spermatozoa in the genital tract after artificial insemination of progestagen-treated ewes. J Reprod Fertil 1969;19:73-86.
10. Lang DR, Oh YK: Distribution of spermatozoa in the reproductive tract of the Romney ewe. N Z J Agric Res 1979;13:157-166.
11. Salamon S, Robinson TJ: Studies on the artificial insemination of Merino sheep. I. The effect of frequency and season of insemination, age of the ewe, rams, and milk diluents on lambing performance. Aust JAgric Res 1962;3:52-68.
12. Colas G: Semen technology in the ram. In: Courot M, editor. The male in farm animal reproduction.. Leiden: Martinus Nijoff Publishers; 1984. p. 219-236.
13. Langford GA, Marcus GJ: Influence of sperm number and seminal plasma on fertility of progestagen treated sheep in confinement. J Reprod Fertil 1982:65:325-329.
14. Bretzlaff KN, Romano JE: Advanced reproductive techniques in goats.Vet Clin North Am Food Anim Pract 2001;17:421-434.
15. Romano JE, Fernandez Abella D, Villegas N: A note on the effect of continuous ram presence on estrus onset, estrus duration and ovulation time in estrus synchronized ewes. Appl Anim Behav Sci 2001;73:193-198.
16. Walker SK, Smith DH, Godfrey B, et al: Time of ovulation in the South Australian Merino ewe following synchronization of estrus. 1. Variation within and between flocks. Theriogenology 1989;31:545-553.

17. Eppleston J, Maxwell WMC: Sources of variation in the reproductive performance of ewes inseminated with frozen-thawed ram semen by laparoscopy. Theriogenology 1995;43:777-778.
18. Findlater RCF, Haresign W, Curnock RM, et al: Evaluation of intrauterine insemination of sheep with frozen semen: effects of time of insemination and semen dose on conception rates. Anim Prod 1991;53:89-96.
19. Romano JE, Rodas E, Ferreira A, et al: Effects of progestagen, PMSG and artificial insemination times on fertility and prolificacy in Corriedale ewes. Small Rumin Res 1996;23:157-162.
20. Romano JE: Comparison of fluorgestone and medroxyprogesterone intravaginal pessaries for estrus synchronization in dairy goats. Small Rumin Res 1996;22:219-223.
21. Romano JE: Effect of two doses of cloprostenol in two schemes for estrous synchronization in Nubian goats. Small Rumin Res 1998;28:171-176.
22. Romano JE: Synchronization of estrus using CIDR, FGA or MAP intravaginal pessaries during the breeding season in Nubian goats. Small Rumin Res 2004;55:15-19.
23. Romano JE: The effect of continuous presence of bucks on hastening the onset of estrus in synchronized does during the breeding season. Small Rumin Res 1998;30:99-103.
24. Romano JE, Christians CJ, Crabo BG.\ : Continuous presence of rams hastens the onset of estrus in ewes synchronized during the breeding season. Appl Anim Behav Sci 2000;66:65-70.
25. Romano JE: Does in proestrus-estrus hasten estrus onset in does estrous synchronized during breeding season. Appl Anim Behav Sci 2002;77:329-334.
26. Romano JE. Effect of service on estrus duration in dairy goats. Theriogenology. 1993;40:77-84.
27. Doney JM, Gin RG, Griffiths JG.The effect of premating stress on the onset of oestrus and on ovulation rate in Scottish Blackface ewes. J Reprod Fertil 1973;35:381-384.
28. Mani AU, McKelvey WAC, Watson ED: The effects of low level of feeding on response to synchronization to oestrus, ovulation rate and embryo loss in goats. Theriogenology 1992;38:1013-1022.
29. Greyling JPC, Van Nierkerk CH: Effect of pregnant mare serum gonadotrophin (PMSG) and route ofadministration after progestagen treatment on estrus and LH secretion in the Boer Goat. Small Rumin Res1990;3:511-516.
30. Corteel JM, Gonzalez C, Nunes JF: Research and development in the control of reproduction. ProcThird Intl Conf Goat Prod Dis; 1982. p. 584-601.
31. Ritar AJ, Ball PD, O'May JO: Artificial insemination of cashmere goats: effects on fertility and fecundity of intravaginal treatment, method and time of insemination, semen freezing process, number of motile spermatozoa an age of females. Reprod Fertil Dev 1990;2:377-384.
32. Maxwell WMC, Hewitt LJ: A comparison of vaginal, cervical and intrauterine insemination of sheep. J Agric Sci 1986;106:191-193.
33. Ritar AJ: Control of ovulation, storage of semen, and artificial insemination of fiber-producing goats in Australia: a review. Aust J Exper Agric 1993;33:807-820.
34. Eppleston J, Salamon S, Moore NW, et al: The depth of cervical insemination and site of intrauterine insemination and their relationship to the fertility of frozen-thawed ram semen. Anim Reprod Sci 1994;36:211-225.
35. Colas G: Fertility in the ewe after artificial insemination with fresh and frozen semen at the induced oestrus, and influence of the photoperiod on the semen quality of the ram. Livest Prod Sci 1979;6:153-166.
36. Windsor DP: Variation between ejaculates in the fertility of frozen ram semen used for cervical insemination of Merino ewes. Anim Reprod Sci 1997;47, 21-29.
37. Söderquist L, Lundeheim N, Nilsson B: Assessment of fertility after using different procedures to thaw ram spermatozoa frozen in ministraws. Reprod Domest Anim 1999;34:61-66.
38. Maxwell WMC: Artificial insemination of ewes with frozen-thawed semen at a synchronized oestrus: effect of time of onset of oestrus, ovulation and insemination on fertility. Anim Reprod Sci 1986;10:301-308.
39. Eppleston J, Roberts EM: The effect of progestagen, PMSG and time of insemination on fertility in ewes following intra-uterine insemination with frozen semen. Aust Vet J 1986;63:124-125.
40. Salamon S, Maxwell WMC: Storage of ram semen. Anim Reprod Sci 2000;62:77-111.
41. Pontbriand D, Howard JG, Schiewe MC, et ala; Effect of cryoprotective diluent and method of freeze-thawing on survival and acrosomal integrity of ram spermatozoa. Cryobiology1989;26:341-354.
42. Nur Z, Zik B, Ustuner B, et al: Effects of different cryoprotective agents on ram sperm morphology and DNA integrity. Theriogenology 2010;73:1267-1275.
43. Hawk HW. Sperm survival and transport in the female reproductive tract. J Dairy Sci 1983;66:2645-2660.
44. Lightfoot RJ, Salamon S: Fertility of ram spermatozoa frozen by the pellet method. I. Transport and viability of spermatozoa within the genital tract of the ewe. J Reprod Fertil 1970;22:385-398.
45. Sullivan JJ: Sperm number required for optimum breeding efficiency in cattle. In: Proc Third Tech Conf AI Reprod; 1972. p. 36-43.
46. Windsor DP: Factors influencing the success of transcervical insemination in Merino ewes. Theriogenology 1995;43:1009-1018.
47. Roy A: Egg-yolk coagulating enzyme in the semen and Cowper's gland of the goat. Nature 1957;179:318.
48. Iritani A, Nishikawa Y: Studies on the egg-yolk coagulating factors in goat semen: II Properties of the coagulating factor and influential conditions for coagulation. Proc Silver Jubilee Lab Anim Husbandry, Kyoto Univesity; 1961. p. 97-104.

49. Nunes JF, Corteel JM, Combarnous Y, et ala; Role of seminal plasma in the in vitro survival of goat sperm. Reprod Nutr Dev 1982;22:611-620.
50. Khalifa T, Lymberopoulos A., Theodosiadou E: Association of soybean-based extenders with field fertility of stored ram (*Ovis aries*) semen: a randomized double-blind parallel group design.Theriogenology 2013;79:517-527.
51 Corteel JM : Viabilité des spermatozoides de bouc conservés et congelés avec ou sans leur plasma seminal: effect du glucose. Ann Biol Anbim Biochm Biophys 1974:14:741-745.
52. Corteel JM. Effect du 'lavage' sur la conservatgion de spermatozoides de bouc a basse temperature. Ann Biol Anbim Biochm Biophys 1977;15:525-528.
53. Ritar AJ, Salamon S, Ball PD, et al: Ovulation and fertility in goats after intravaginal device-PMSG treatment. Small Rumin Res 1989;2:323-331.
54. Memon MA, Bretzlaff KN, Ott RS. Effect of washing on motility and acrosome morphology of frozen-thawed goat spermatozoa. Am J Vet Res 1985;46:473-475.
55. Roof DS, Bowley S, Price LL, et al: Comparison of two commercial extenders for cryopreservation of goat semen without sperm washing. Theriogenology 2012;77:412-420.
56. Loubser PG, Greyling JPC, Viljoen KS: Artificial insemination of Angora goat does with pelleted deep-frozen semen. S Afr J Anim Sci 1983;13:134-135.
57. Mattner PE, Entwistle KW, Martin ICA: Passage, survival, and fertility of deep-frozen ram semen in the genital tract of the ewe. Aust J Biol Sci 1969;22:181-187.
58. Hawk HW: Transport and fate of spermatozoa after insemination of cattle. J Dairy Sci 1987;70:1487-1503.
59. Salamon S. Fertility following deposition of equal numbers of frozen-thawed ram spermatozoa by single or double insemination. Aust J Agric Res 1977;28:477-479.
60 Lightfoot RJ, Salamon S: Fertility of ram spermatozoa frozen by the pellet method. II. The effects of method of insemination on fertilization and embryonic mortaltiy. J Reprod Fertil 1970;22:399-408.
61. Bunch TD, Elssworth HS: Gross anatomy of the ovine cervix. Int Goat Sheep Res 1981;1:282-285.
62. More J: Anatomy and histology of the cervix uteri of the ewe: new insights. Acta Anat 1984;120:156-159.
63. Reinhold VG, Rommel W, Schultz J: Untersuchungen zum anatomischen Aufbau er Cervix uteri des Merinofleishshafes unter derm Aspekt der künstlichen Besamung. Mh Vet Med 1987;42:364-368.
64. Halbert GW, Dobson H, Walton JS, et al: The structure of the cervical canal of the ewe. Theriogenology 1990;33:977-992.
65. Kershaw CM, Khalid M, McGowan MR,et al: The anatomy of the sheep cervix and its influence on the transcervical passage of an inseminating pipette into the uterine lumen. Theriogenology 2005;641225-1235.
66. Andersen V, Aamdal J, Fougner J: Interuterine and deep cervical insemination with frozen semen in sheep. Zuchthygiene 1973;8:113-118.
67. Fukui Y, Roberts EM: Further studies on non-surgical intrauterine technique for artificial insemination in the ewe. Theriogenology 1978;10:381-393.
68. Olaffson T: Insemination of sheep with frozen semen: results obtained in a field trail in Norway. Zuchthgyene 1980;15:50-59.
69. Halbert GW, Dobson H, Walton JS, et al: A technique for trasncervical intrauterine insemination of ewes. Theriogenology 1990;33:993-1010.
70. Buckrell BC, C. Buschbeck, C.J. Gartley, et al: Further development of a transcervical technique for artificial insemination in sheep using previously frozen semen. Theriogenology 1994;42:601-611.
71. Windsor DP, Széll AZ, Buschbeck C, et al: Transcervical artificial insemination of Australian Merino ewes with frozen-thawed semen. Theriogenology 1994;42:147-157.
72. Alvarez M, Chamorro CA, Kaabi M, et al: Design and "in vivo" evaluation of two adapted catheters for intrauterine transcervical insemination in sheep. Anim Reprod Sci 2012;131:153-159.
73. Lawrence R: Preliminary results of non-surgical intrauterine insemination of sheep with thawed semen. J S Afr Vet Assoc 1985:56:61-63.
74. Husein MQ, Bailey MT, Ababneh MM, et al: Effect of eCG on the pregnancy rate of ewes transcervically inseminated with frozen-thawed semen outside the breeding season. Theriogenology 1998;49:997-1005.
75. Husein MQ, Romano JE, Bailey MT, et al: Estrous synchronisation and pregnancy rate of transcervically inseminated ewes during the breeding season. Sheep Goat Res J 1998;14:148-152.
76. Smith JF, Parr J, Beaumont S, et al: Comparison of cervical, transcervical and laparoscopic insemination of ewes with chilled, stored and frozen ram semen. Proc NZ Soc Anim Prod 1995;55:248-250.
77. Campbell JW, Harvey TG, McDonald MF, et al: Transcervical insemination in sheep: an anatomical and histological evaluation. Theriogenology 1996;45:1535-1544.
78. Halbert GW, Dobson H, Walton JS, et al: Field evaluation of a technique for trasncervical intrauterine insemination of ewes. Theriogenology 1990;33:1231-1243.
79. McKelvey WAC: Recent research on AI and MOET in sheep and their commercial application in the UK. Proc NZ Embryo Transfer Workshop 1994. p. 16-17.
80. Salamon S, Lightfoot RJ: Fertilization and embryonic loss in sheep after insemination with deep frozen semen. Nature 1967;216:194-195.
81. Killeen ID, Caffery GJ: Uterine insemination of ewes with the aid of a laparoscope. Aust Vet J 1982;59:95.

82. Fieni F, Buggin M, Tainturier D, et al: L'insemination artificielle intra-utérine, transpéritonéal chez la chèvre. Rec Med Vet 1990;166:471-478.
83. Vallet JC, Baril G, Leboeuf B, et al: Insémination artificielle intra-utérine sous contrôle laparoscopique chez les petits ruminants domestiques. Ann Zootech 1992;41:305-309.
84. Maxwell WMC: Artificial insemination of ewes with frozen-thawed semen at a synchronized oestrus: effect of dose of spermatozoa and site of intrauterine insemination on fertility. Anim Reprod Sci 1986;10:309-316.
85. Gourley DD, Riese RL: Laparoscopic artificial insemination in sheep. Vet Clin North Am Food Anim Pract. 1990;6:615-633.
86. Leboeuf B, Nercy C, De Ruyter T : L'inseminatin artificielle caprine au Rwanda. Adaptation à la chèvre rwandaise de la méthode utilisée pour les races laitières européennes. Revue Elev Med Vet Pays Trop 1994;47:240-243.
87. Moore RW. Miller CM, Hall DRH, et al: Cervical versus laparoscopic AI in goats after PMSG injection at or 48 hours before CIDR removal. Proc New Zeal Soc Anim Prod 1998;48:69-70.
88. Fieni F, Buggin M, Tainturier D, et al: Study of the best hour for intrauterine insemination in young dairy goats after hormonal induction of oestrus. Theriogenology 1991;35:200 (abstract).
89. Armstrong DT, Evans G: Intrauterine insemination enhances fertility of frozen semen in superovulated ewes. J Reprod Fertil 1984;71:89-94.
90. Scudamore CL, Robinson JJ, Aitken RP, et al: Laparosocpy for intrauterine insemination and embryo recovery in superovulated at a commercial embryo transfer unit. Theriogenology 1991;35-329-337.
91. Warwick BL, Berry RO, Horlacher WR: Results of mating rams to Angora female goats. Proc 27th Annu Meet Am Soc Anim Prod 1934;27:225-227.
92. Warwick BL, Berry RO: Inter-generic and intra-specific embryo transfers in sheep and goats. J Hered 1949;40:297-303.
93. Kraemer DC: Embryo collection and transfer in small ruminants. Theriogenology 1989;31:14-148.
94. Cognié Y, Baril G, Poulin N, et al: Current status of embryo technologies in sheep and goat. Theriogenology 2003;59:171-188.
95. Armstrong DT, Evans G: Factors influencing success of embryo transfer in sheep and goats. Theriogenology 1983;19:31-42.
96. Cognie Y: State of the art in sheep-goat embryo transfer. Theriogenology 1999;51:105-116.
97. Baldassarre H, Karatzas CN: Advanced assisted reproduction technologies (ART) in goats. Anim Reprod Sci 2004;82-83:255-266.
98. Stringfellow DA, Seidel SM: Manual of the International Embryo Transfer Society. 3rd edition.Champaign(IL): International Embryo Transfer Society; 1998. p. 174.
99. Armstrong DT, Pfitzner AP, Warnes GM, et al: Superovulation treatments and embryo transfer in Angora goats. J Reprod Fertil 1983;67:403-410.
100. Pendleton RJ, Youngs CR, Rorie RW, et al: Follicle stimulating hormone versus pregnant mare serum gonadotropin for superovulation of dairy goats. Small Rumin Res 1992;8:217-224.
101. Batt PA, Killeen ID, Cameron WN: Use of single or multiple injections of FSH in embryo collecdtion programmes in goats. Reprod Fertil Dev 1993;5:49-56.
102. Roy F, Combes B, Vaiman D, et al: Humoral immune response to equine chorionic gonadotropin in ewes: association with major histocompatibility complex and interference with subsequent fertility. Biol Reprod 1999;61:209-218.
103. Roy F, Maurel MC, Combes B, et al: The negative effect of repeated equine chorionic gonadotropin treatment on subsequent fertility in Alpine goats is due to a humoral immune response involving the major histocompatibility complex. Biol Reprod 1999;60:805-813.
104. Martemucci G, D'Alessandro A, Toteda F, et ala; Embryo production and endocrine response in ewes superovulated with PMSG, with or without monoclonal anti-PMSG administered at different times. Theriogenology 1995;44:691-703.
105. Pintado B, Gutiérrez-Adán A, Pérez Llano B: Superovulatory response of Murciana goats to treatments based on PMSG/anti-PMSG or combined FSH/PMSG administration. Theriogenology 1998;50:357-364.
106. Medan MS, Watanabe G, Sasaki K, et al: Ovarian and hormonal response of female goats to active immunization against inhibin. J Endocrinol 2003;177:287-294.
107. Hunter GL, Adams CE, Rowson LE: Inter-breed ovum transfer in sheep. J Agric Sci 1955;46:143-149.
108. Tervit HR, Havik PG: A modified technique for flushing ova from the sheep uterus. N Z Vet J 1976;24:138-140.
109. Torres S, Sevellec C: Repeated superovulation and surgical recovery of embryos in the ewe. Reprod Nutr Develop 1987;27:859-863.
110. Baril G, Brebion P, Chesné P: Manuel de formation pratique pour la transplantation embrionnaire chez la brebis et al chèvre. FAO 115. Rome: Food and Agriculture Organization of the United Nations; 1993. p. 185.
111. Seeger K, Klatt PR: Laparoscopy in the sheep and goat. In: Harrison RM, Wildt DE, eds. Animal laparoscopy. Baltimore: Williams and Wilkins; 1980. p. 107-120.
112. Mckelvey WAC, Robinson JJ, Aitken RP, et al: Repeated recoveries of embryos from ewes by laparoscopy. Theriogenology 1986;25:855-865.
113. Legendre X, Bousseau S, Koyundjiev S: Embryo collection from ewes by laparoscopy using a 3-way rigid catheter (abstract). Theriogenology 1988;29:269.

114. Nellenshulte E, Niemann H: Collection and transfer of ovine embryos by laparoscopy. Anim Reprod Sci 1992;27:293-304.
115. Bondurant RH, Skirrow S, Anderson GB, et al: Non-surgical collection of blastocyst from dairy goats. Theriogenology 1984;22:423-431.
116. Van Niekerk CH, Barry DM, Rust JM, et al: Cervical softening with prostaglandin E2 and estradiol cypionate for embryo collection in goats (abstract). Theriogenology 1990;33:348.
117. Flores-Foxworth G, McBride BM, Kraemer DC, et al: A comparison between laparoscopic and transcervical embryo collection and transfer in goats (abstract). Theriogenology 1992;37:213.
118. Pereira RJ, Sohnrey B, Holz W: Nonsurgical embryo collection in goats treated with prostaglandin F2alpha and oxytocin. J Anim Sci 1998;76:360-363.
119. Coonrod SA, Coren BR, McBride BL, et al: Successful non-surgical collection of ovine embryos (abstract). Theriogenology 1986;25:149.
120 Barry DM, van Niekerk CH, Rust J,et al: Cervical embryo collection in sheep after "ripening of the cervix with prostaglandin E2 and estradiol (abstract). Theriogenology 1990;33:190.
121. Mylne MJA, McKelvey WAC, Fernie K, et al: Use of transcervical technique for embryo recovery in sheep. Vet Rec 1992;130:450-451.
122. Jackson P: ET procedures in goats. Proc Embryo Transfer in Sheep and Goats. Refresher Course for Veterinarians; 1993. p. 57-67.
123. Mutiga ER, Baker AA: Transfer of sheep embryos through a laparoscope. Vet Rec 1984;114:401-402.
124. McKelvey WAC, Robinson JJ, Aitken RP: A simplified technique for transfer of ovine embryos by laparoscopy. Vet Rec 1985;117:492-494.
125. Schiewe MC, Bush M, Stuart LS, et al: DE. Laparoscopic embryo transfer in domestic sheep: a preliminary study. Theriogenology 1984;22:675-682.
126. Moore NW, Shelton JN: Egg transfer in sheep. Effect of degree of synchronization between donor and recipient, age of egg, and site of transfer on the survival of transferred eggs. J Reprod Fertil 1964;7:145-152.
127. Willadsen SM: A method for culture of micromanipulated sheep embryos and its use to produce monozygotic twins. Nature 1979;277:298-300.
128. Bretzlaff KN, Weston PG, Hixon JE, et al: Plasma luteinizing hormone and progesterone concentrations in goats with estrous cycles of normal or short duration after prostaglandin F2 alpha administration during diestrus or pregnancy. Am J Vet Res 1988;49:939-943.
129. Cervantes MJ, Juárez ML, Mejía VO, et al: Use of fluorogestone acetate after breeding to reduce the effect of premature luteal regression in dairy goats when superovulation is induced with FSH. Anim Reprod Sci 2007;97:47-54.
130. Battye KM, Fairclough RJ, Cameron AW, et al: Evidence for prostaglandin involvement in early luteal regression of the superovulated nanny goat (*Capra hircus*). J Reprod Fertil 1988;84:425-430.
131. Saharrea A, Valencia J, Balcázar A, et al: Premature luteal regression in goats superovulated with PMSG: effect of hCG or GnRH administration during the early luteal phase. Theriogenology 1998;50:1039-1052.
132. Rao VH, Shamah BC, Bhattacharyya NK: Xenogenous fertilization of goat ova in the rabbit oviduct. J Reprod Fertil 1984;71:377-379.
133. Hanada A: In vitro fertilization in goats. Jap J Anim Reprod 1985;31:21-26.
134. Younis AI, Zuelke KA, Harper KM, et al: In vitro fertilization of goat oocytes. Biol Reprod 1991;44:1177-1182.
135. Crozet N, De Smedt V, Ahmed-Ali M, et al: Normal development following in vitro oocyte maturation and fertilization in the goat (abstract). Theriogenology 1993;39:206.
136. Keskintepe L, Darwish GM, Kenimer AT, et al: Term development of caprine embryos derived from immature oocytes in vitro. Theriogenology 1994;42:527-535.
137. De Smedt V, Crozet N, Ahmed-Ali M, et al: In vitro maturation and fertilization of goat oocytes. Theriogenology 1992;37:1049-1060.
138. Todini L, Cognie Y, Poulin N, Guerin Y. Recupero in vivo dei oocittii de capra mediante aspirazione folliculare per aspiration. Atti XI Cong Nat. Perugia, Italy; 1994. p. 331-334.
139. Graff KJ, Meintjes M, Dyer VW, et al: Transvaginal ultrasound-guided oocyte retrieval following FSH stimulation of domestic goats. Theriogenology 1999;51:1099-119.
140. Martino A, Mogar T, Palomo MJ, et al: Meiotic competence of prepubertal goat oocytes. Theriogenology 1994;42:859-873.
141. Lacalandra GM, Minoia P, Dell'Aquila ME, et al: Surgical recovery of oocytes from follicles of pregnant goats. Proc 11th Int Cong Anim Reprod AI; 1992. p. 169-171.
142. Pawshe CH, Totey SM, Jain SK: A comparison of three methods of recovery of goat oocytes for in vitro maturation and fertilization. Theriogenology 1994;42:117-126.
143. Le Gal F: In vitro maturation and fertilization of goat oocytes frozen at the germinal vesicle stage. Theriogenology 1996;45:1177-1185.
144. Pawshe CH, Jain SK, Totey SM: Effecdt of commercial available follicle stimulating hormone on in vitro maturation of goat oocytes. Indian J Anim Reprod 1993;14:69-71.
145. Pawshe CH, Palanisamy A, Taneja M, et al: Comparison of various maturation treatments on in vitro maturation of goat oocytes and their early embryonic development and cell numbers. Theriogenology 1996;46:971-982.

146. Cox JF, Saravia F, Briones M, et al: Dose-dependent effect of heparin on fertilizing ability of goat spermatozoa. Theriogenology 1995;44:451-460.
147. Cox JF, Catalan A, Saravia F, et al: In vitro fertilization of cattle and sheep oocytes by goat spermatozoa. Small Rumin Res 1994;15:55-58.
148. Ling L, Jufren A, Yong Z: In vitro fertilization of goat ovarian oocytes (abstract). Theriogenology 1992:37:247.
149. Keskintepe L, Darwish GM, Younis AI, et al: In vitro development of morulae from immature caprine oocytes. Zygote 1994;2:97-102.
150. Huang JC, Wen SH, Shie RC, et al: In vitro fertilization and early development of caprine oocytes and embryos. J Taiwan Livest Res 1989;22:39-46.
151. Duan EK, Wang GY, Ma BH, et al: Xenogenous fertilization of goat ovulated oocytes in the rabbit oviduct (abstract). Theriogenology 1990;33:219.
152. Tibary A, Anouassi A, Khatir H: Update on reproductive biotechnologies in small ruminants and camelids. Theriogenology 2005;64:618-638.
153. Cognié Y, Poulin N, Baril G, et al: Embryo survival after transfer of in vitro and in vivo produced goats embryos (abstract). Proc 17th Meet Assoc Eur Trans Emb (AETE); 2001. p. 110
154. Gandolfi F, Moor RM: Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. J Reprod Fertil 1987;81:23-28.
155. Brown B, Radziewic T: Production of sheep embryos in vitro and development of progeny following single and twin embryo transfers. Theriogenology 1998;49:1525-1536.
156. Leibo SP: Cryopreservation of oocytes and embryos: optimization by theoretical versus empirical analysis.Theriogenology 2008;69:37-47.
157. Uehara T, Yanagimachi R: Microsurgical injection of spermatozoa into hamster eggs with subsequent transformation of sperm nuclei into male pronuclei. Biol Reprod 1976;15:467-470.
158. Catt SL, Catt JW, Gomez MC, et al: Birth of a male lamb derived from an in vitro matured oocyte fertilized by intracytoplasmic injection of a single presumptive male sperm. Vet Rec 1996;16:494-495.
159. Keskintepe LPC, Morton SE, Smith MJ, et al: Caprine blastocyst formation following intracytoplasmic sperm injection and defined culture. Zygote 1997;5:261-265.
160. Wang B, H. Baldassarre J, Pierson F, et al: The in vitro and in vivo development of goat embryos produced by intracytoplasmic sperm injection using tail-cut spermatozoa. Zygote 2003;11:219-227.
161. López-Saucedo J, Paramio-Nieto MT, Fierro R, et al: Intracytoplasmic sperm injection (ICSI) in small ruminants. Anim Reprod Sci 2012;133:129-138.
162. Shin T, Long C, Foxworth G, et al: A simple modified ICSI technique resulting in high pregnancy rates in Merino sheep (*Ovis aries*). Biol Reprod 2005;73(suppl):234.
163. Bormann C, Long C, Menges S, et al: Reestablishment of an extinct strain of sheep from a limited supply of frozen semen. Reprod Fertil Dev 2006;18:201-202.
164. Trounson AO, Moore NW: Attempts to produce identical offspring in the sheep by mechanical division of the ovum. Aust J Biol Sci 1974;27:505-510.
165. Vajta G, Gjerris M: Science and technology of farm animal cloning: state of the art. Anim Reprod Sci 2006;92:211-230.
166. Gatica R, Boland MP, Crosby TF, et al: Micromanipulation of sheep morulae to produce monozygotic twins. Theriogenology 1984;21:555-560.
167. Willadsen SM: The viability of early cleavage stages containing half the normal number of blastomeres in the sheep. J Reprod Fertil 1980;59:357-362.
168. Willadsen SM: Nuclear transplantation in sheep embryos. Nature 1986;320:63-65.
169. Smith LC, Wilmut I: Influence of nuclear and cytoplasmic activity on the development in vivo of sheep embryos after nuclear transplantation. Biol Reprod 1989;40:1027-1035.
170. Wilmut I, Schnieke AE, McWhir J, et al: Viable offspring derived from fetal and adult mammalian cells. Nature 1997; 385:810-813.
171. Baguisi A, Behboodi E, Melican DT,et al: Production of goats by somatic cell nuclear transfer. Nat Biotechnol 1999;17:456-461.
172. Niemann H, Lucas-Hahn A: Somatic cell nuclear transfer cloning: practical applications and current legislation. Reprod Domest Anim 2012;47:2-10.
173. McLaughlin KJ, Davies L, Seamark RF: In vitro embryo culture in the production of identical merino lambs by nuclear transplantation. Reprod Fertil Dev 1990;2:619-622.
174. Liu J, Westhusin M, Long C, et al: Embryo production and possible species preservation by nuclear transfer of somatic cells isolated from bovine semen. Theriogenology 2010;74:1629-1635.
175. Ebert KM, Selgrath JP, DiTullio P, et al: Transgenic production of a variant of human tissue-type plasminogen activator in goat milk: generation of transgenic goats and analysis of expression. Biotechnology (NY). 1991;9:835-838.
176. Yekta A, Dalman A, Efterkhari-Yadi P, et al: Production of transgenic goats expressing human coagulation factor IX in the mammary gland after nuclear transfer using transfected fetal fibroblast cells. Transgenic Res 2013;22:131-142.
177. Zhang YL, Wan YJ, Wang ZY, et al: Production of dairy goat embryos, by nuclear transfer, transgenic for human acid beta-glucosidase. Theriogenology 2010;73:681-690.

178. Seidel GE, Garner DL: Current status of sexing mammalian spermatozoa. Reproduction 2002;124:733-743.
179. Beilby KH, Grupen CG, Thomson PC, et al: The effect of insemination time and sperm dose on pregnancy rate using sex-sorted ram sperm. Theriogenology. 2009;71:829-835.
180. de Graaf SP, Beilby K, O'Brien JK, et al: Embryo production from superovulated sheep inseminated with sex-sorted ram spermatozoa.Theriogenology. 2007;67:550-555.
181. Beilby KH, de Graaf SP, Grupen CG: The effect of sperm and cryoprotectant concentration on the freezing success of sex sorted ram sperm for in vitro fertilization. Theriogenology 2010;74:786-794.
182. de Graaf SP, Beilby K, Underwood SL, et al: Sperm sexing in sheep and cattle: the exception and the rule. Theriogenologoy. 2009;71:89-97.
183. Bredenoord AL, Pennings G, de Wert G: Ooplasmic and nuclear transfer to prevent mitochondrial DNA disorders: conceptual and normative issues. Hum Reprod Update 2008;14:669-678.

Can pain affect reproduction?

L. Chris Sanchez
Department of Large Animal Clinical Sciences, University of Florida, Gainesville, FL

Abstract

Subtle pain can be challenging to recognize clinically. Very little work has been done to critically evaluate the effect of pain on reproductive performance in horses. A small body of evidence suggests that lameness has a negative impact on reproductive performance in dairy cattle. This talk will discuss recognition of subtle pain in horses and how both musculoskeletal and visceral pain may have an impact on fertility.

Keywords: Mare, pain scoring

Overview of pain in horses

Most horse owners and veterinarians are comfortable recognizing signs of moderate to severe pain in horses, with colic and lameness being the most commonly recognized forms of visceral and somatic pain, respectively. But, more subtle signs of pain can be challenging to recognize. Whether pain is recognized or not, chronic pain may have substantial systemic effects and, in some cases, may result in either infertility or subfertility.

In human beings capable of verbal communication, pain is typically evaluated by self-assessment. In animals and non-verbal humans, pain assessment is always based upon the perception of an observer, be that an owner, trainer, or veterinarian. Thus, when addressing an animal's status, inherent differences in based upon species, age, sex, genetics, environment, source (visceral, somatic, neuropathic), and duration (acute versus chronic) can affect one's perception of pain. When evaluating pain in horses, careful evaluation of the animal in its environment can provide useful information.

One way to summarize subtle signs of pain in horses is to evaluate the factors included in some of the more commonly used clinical pain scoring systems. These include the animal's time spent in various locations within the stall (head in a corner, looking out a window, at the front of the stall, etc.), in various positions or activities (ear position, head position, eating, lying down, etc.) or performing various events (vocalizing, stomping feet, shifting weight, etc.). Time budget and event analyses have been able to detect differences in post-operative pain assessment following colic and arthroscopic surgery.[1,2] Using a numerical rating system, a Washington State group has shown that horses receiving a butorphanol constant rate infusion in addition to flunixin following colic surgery had improved behavior/pain scores in the 24 hours following surgery, decreased cortisol concentration, less weight loss, and faster discharge from the hospital relative to horses receiving flunixin and a placebo infusion.[3] Similar results have been found in humans, where treatment by an organized pain service results in lower postoperative pain scores, improved satisfaction, and accelerated hospital discharge.[4]

Pain and reproductive performance in other species

A rise in nociceptive or pain threshold has been documented in pigs and humans during late gestation and labor, which is reversed post-partum.[5,6] Some evidence suggests that this is an opioid-mediated event as it can be reversed with naloxone in sows.[5] Hormonal factors likely play a role in this period of hypoalgesia in addition to neuronal factors, as 17-beta estradiol and progesterone modulate an opioid analgesic system during pregnancy.[7]

Much of the work demonstrating an inversely proportional relationship between pain and fertility has been performed in dairy cattle. Lameness has a substantial impact on not only the welfare of dairy cows, but also the bottom line for producers. In a Florida cohort study of 837 dairy cows, claw lesions were the most common cause of lameness; these lesions were associated with a longer (40 days) time to conception) and a higher number of breedings per conception relative to healthy cows.[8] Similar effects of lameness have been shown on other measures of reproductive performance.[9,10]

Because stress stimulates the hypothalamic-pituitary-adrenal (HPA) axis, one assumes that lameness, and by association, other forms of acute and/or chronic pain can therefore disrupt the hypothalamic-pituitary-ovarian axis and its associated hormonal release, thereby disrupting reproductive function. In an attempt to further refine this hypothesis, 59 dairy cattle were observed following estrus synchronization with gonadotropin releasing hormone followed by prostaglandin $F_{2\alpha}$ seven days later.[11] In that study, lame cows had lower estrus intensity scores and lower milk progesterone concentrations in the six days prior to estrus but no difference in duration of estrus or milk estradiol or cortisol concentrations relative to healthy cows. The authors concluded that lame cows may be related to decreased sexual or "stress-related" pheromones or processing thereof. This confirmed the authors' prior work in which prior progesterone, but not estradiol, concentrations were associated with lameness and estrus behavior.[11,12]

One can certainly infer that, if acute and/or chronic pain substantially affects reproductive performance in one species, similar effects are likely to occur in other species as well. The literature profiling pain in horses is remarkably small, though the vast majority describes lameness and causes thereof. Thus, it is not particularly surprising that an association between pain and fertility – or infertility – has not yet been established in the horse. The lack of data, however, certainly does not infer lack of relationship.

References

1. Pritchett LC, Ulibarri C, Roberts MC, et al: Identification of potential physiological and behavioral indicators of postoperative pain in horses after exploratory celiotomy for colic. Appl Anim Behav Sci 2003;80:31-43.
2. Price J, Catriona S, Welsh EM, et al: Preliminary evaluation of a behaviour-based system for assessment of post-operative pain in horses following arthroscopic surgery. Vet Anaesth Analg 2003;30:124-137.
3. Sellon DC, Roberts MC, Blikslager AT, et al: Effects of continuous rate intravenous infusion of butorphanol on physiologic and outcome variables in horses after celiotomy. J Vet Intern Med 2004;18:555-563.
4. Miaskowski C, Crews J, Ready LB, et al: Anesthesia-based pain services improve the quality of postoperative pain management. Pain 1999;80:23-29.
5. Jarvis S, McLean KA, Chirnside J, et al: Opioid-mediated changes in nociceptive threshold during pregnancy and parturition in the sow. Pain 1997:153-159.
6. Ohel I, Walfisch A, Shitenberg D, et al: A rise in pain threshold during labor: a prospective clinical trial. Pain 2007;132 Suppl 1:S104-S108.
7. Dawson-Basoa MB, Gintzler AR: 17-Beta-estradiol and progesterone modulate an intrinsic opioid analgesic system. Brain Res 1993;601:241-245.
8. Hernandez J, Shearer JK, Webb DW: Effect of lameness on the calving-to-conception interval in dairy cows. J Am Vet Med Assoc 2001;218:1611-1614.
9. Garbarino EJ, Hernandez JA, Shearer JK, et al: Effect of lameness on ovarian activity in postpartum Holstein cows. J Dairy Sci 2004;87:4123-4131.
10. Hernandez JA, Garbarino EJ, Shearer JK, et al: Comparison of the calving-to-conception interval in dairy cows with different degrees of lameness during the prebreeding postpartum period. J Am Vet Med Assoc 2005;227:1284-1291.
11. Walker SL, Smith RF, Jones DN, et al: The effect of a chronic stressor, lameness, on detailed sexual behaviour and hormonal profiles in milk and plasma of dairy cattle. Reprod Domest Anim 2010;45:109-117.
12. Walker SL, Smith RF, Jones DN, et al: Chronic stress, hormone profiles and estrus intensity in dairy cattle. Horm Behav 2008;53:493-501.

An introduction to acupuncture and its incorporation into equine reproductive practice

Michelle LeBlanc,[a] Allison Faber[b]
[a]Rood and Riddle Equine Hospital, Lexington, KY; [b]Full Circle Veterinary Services, Mechanicsville, VA

Introduction

Traditional Chinese veterinary medicine (TCVM) has been used to treat animals, primarily horses, cattle and pigs, for thousands of years in China. It differs from Western medicine in that acupuncture focuses on "balance" of the physiological, spiritual and emotional wellbeing of an individual while conventional Western medicine focuses on controlling a disease. Western medical practitioners analyze a disease to discover its specific, physical cause, whether this is an infectious agent, an enzymatic defect, cancer or a toxic insult. By fully understanding the functions of the physical body down to a cellular or molecular level, one can target the abnormality and better control the disease process.[1]

On the other hand TCVM practitioners recognize disease as an imbalance in the body. They understand that the body is an integrated, energetic structure and that disturbance of energy flow creates disease in the whole organism. When a disease pattern is identified, one can restore balance and health by helping the body regulate itself. Traditional Chinese veterinary medicine therapy is directed at not only treating the disease but identifies external and internal factors contributing to the primary abnormality. These include environment, nutrition, hereditary influences, the neuroendocrine system and the animal's emotional state. Therefore, TCVM is directed at evaluating the entire animal and its response to its environment as a whole as each contributes to the imbalance.

Other than pain relief, acupuncture does not directly address any specific clinical sign but normalizes physiological homeostasis and promotes self-healing through normal endogenous pathways. Thus, acupuncture, in terms of its therapeutic mechanisms, is non-specific in that it treats certain tissues through overall body systems and targets balance. As a physiological therapy, the efficacy of acupuncture depends on the pathology involved and the healing potential intrinsic to each patient.[2] Research has shown that the response to acupuncture when used for pain control differs between humans. About 28% are excellent responders, 64% are good and average responders and 8% are weak or non-responders.[3] It is possible that the horse population may respond in a similar manner.

Both Western medicine and TCVM rely on medical history and physical examination to make a diagnosis or identify a pattern. Western medicine adds diagnostic tests such as bloodwork or radiographs. Traditional Chinese veterinary medicine uses tongue color, carotid pulse quality, and pain over acupoints to diagnose an imbalance. The technique takes practice to be able to discern normal from abnormal. Besides the obvious challenges with learning new diagnostic standards, the concepts around which these tools are used are very foreign to our scientifically- and physiologically-based physical examinations. In an oversimplification for purposes of this manuscript, TCVM practitioners look for carotid pulses weaker on the right or left side of the body, and differences in tongue color to diagnose different patterns of energy imbalance. Body temperature also plays a factor in diagnosis, and is usually assessed by ear tip temperature and whole-body (especially lumbar area) palpation. The following is an abbreviated introduction to basic concepts of acupuncture and how it can be incorporated into a reproductive practice.

Meridians and acupoints

Traditional Chinese veterinary medicine is based on energy freely flowing through specific channels called meridians. There are many channels of energy both inside and outside the body, but the most basic consists of 12 channels on the lateral aspects of the body, with an additional two coursing through the dorsal and ventral midlines, totaling 14. Each of the 12 bilateral channels corresponds with an organ, and many organs have relationships with other tissues. Many of the channels on the inside of the body have a connection with the outside through surface acupoints. Recent scientific studies have shown that these connections exist and are the basis for the somatic-visceral reflexes and referred pain. The internal organs are referred to as Zang-Fu organs, with the Fu (Yang) organs being hollow, paired with a Zang (Yin) organ which is structurally solid. Each organ has an association point on the Bladder meridian and these acupoints are commonly used to treat organ dysfunction.

Acupuncture is the stimulation of specific pre-determined points (acupoints) located on meridians near the surface of the body which produces a therapeutic effect by evoking homeostatic mechanisms within the nervous, immune, endocrine, cardiovascular and other body systems to promote self-healing.[2] Acupoints range in size from 1 to 25 mm and can be identified by their electrical conductivity which differs from surrounding tissue. Electrical conductivity of acupoints is higher and electrical resistance is lower than surrounding tissues. At each point is a high density of free nerve endings, arterioles, lymphatic vessels and mast cells. Each acupoint has a defined and specific function. Treatment effects are based on acupoints selected, method of stimulation – dry needles, electro-acupuncture (EA), aquapuncture, or hemo-acupuncture – the length of stimulation and the timing and number of treatments. Some points may be used singly, but it is more common to use several points treated simultaneously, to achieve the desired effect. A typical treatment may involve the use of as few as one to as many as 20 acupoints. Research in humans and rats indicates that stimulation of specific acupoints results in increased blood flow to certain organs, increased movement of lymph, specific changes in the brain as observed on functional magnetic resonance imaging (fMRI), modulation of the immune system and release of opioids from the hypothalamus.

Different types of acupoints can be used for both diagnostic and treatment purposes. Association points or shu points are the most frequently used points for acupuncture diagnosis. They are located on the bladder meridian along the back and each is associated with a certain organ. Part of a TCVM examination involves scanning these diagnostic points to identify areas of pain or sensitivity. Pain when light pressure is placed on a point indicates an acute condition while pain with deep pressure indicates a chronic condition. About 70% of acupoints that exhibit sensitivity correspond to trigger points. Trigger points are defined as circumscribed hypersensitive foci in myofascial structures that give rise to a larger area of pain in adjacent or distant referred areas when palpated. Sensitive acupoints appear to be regions of hyperalgesia that arise spontaneously from local irritation or from excitation of somatic or visceral structures distant from the painful point.[4] As each organ corresponds to a specific bladder acupoint on the horse's back, palpation and elicitation of pain on these specific points indicate either a chronic condition of the musculoskeletal system or a specific problem with a visceral organ.

Neurophysiology

In the last decade, studies on neural mechanism underlying acupuncture analgesia focus on cellular and molecular substrates and functional brain imaging. Needle insertion stimulates afferent A-delta nociceptive fibers which leads to local, spinal cord and brainstem effects. Electro-acupuncture has profound effects on the hypothalamic-pituitary axis.[5-8] The hypothalamic-pituitary axis not only produces the well-known neuroendocrine effects, but it is part of the central descending pain-inhibitory pathways involving endogenous opioids and most likely also plays a role in cholinergic anti-inflammatory mechanisms through the vagus nerve.[9] In studies comparing EA with dry-needle acupuncture, EA produced greater brain changes on fMRI than dry needles and also elicited a better analgesic effect.[2] Electro-acupuncture was more effective than dry-needle acupuncture in activating the release of circulating plasma concentrations of beta endorphins into the cerebrospinal fluid of horses. This effect is most likely associated with stimulation of the hypothalamus and release of beta endorphins from the pituitary.[10] Beta endorphin while implicated in pain control, also has other systemic effects that may correlate with some of the findings seen with acupuncture. Beta-endorphin receptors are present on blood vessels and may contribute to the vasodilatation observed with acupuncture. They are also present in the gut and can alter motility.

Opioid peptides released by EA are frequency-dependent and differ from that released by dry-needle acupuncture. At low frequencies of 4 and 0.2 Hz for 20 minutes on select acupoints, beta endorphins are released whereas at high frequency of 200 Hz serotonin is released. Electro-acupuncture stimulation of certain acupoints associated with reproduction alters plasma levels of oxytocin, prolactin, follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol and progesterone in sheep.[11] Although the hypothalamic-pituitary axis is the major orchestrator of reproductive cyclicity in the mare and semen production in the stallion, and stimulation of the axis results in the release of many

neuroendocrine substances, little is known about how acupuncture interacts and "balances" reproductive function in the horse.

Medical diagnosis

In TCVM a diagnosis is made by collecting clinical data that includes a thorough history followed by evaluating the tongue, pulse, general appearance, body temperature, secretions and excretions, palpation of acupoints, and "shen".[12] Shen refers to the outward manifestations of the vital activities of the horse. It includes evaluation of the animal's eyes (glitter), ears (responds to environmental factors), position of the head, and response to the environmental stimuli. Tongue assessment includes evaluation of color, shape, and coating. Normal tongues are pink, not fat or swollen, and with a normal amount of moisture, not excessively wet. A pale, slightly wet and swollen tongue may be observed in older broodmares with delayed uterine clearance. A red tongue with a thick white coating indicates a heat pattern and may be observed in cases of metritis, inflammation or fever. Whereas a red tongue with no coating indicates a deficient heat pattern, which may be seen in cases with chronic inflammation or infection. Carotid pulse assessment is difficult to learn. It assesses the strength or quality of the pulses both superficially and deep, from right to left. Pulses are obtained by placing three fingers on the right and left carotid arteries at the base of the jugular groove of the horse. Each of the six sites corresponds to two of the 12 meridians and therefore correlates to a specific internal organ.[12] Normal pulses are, of course, even in all six areas and from side to side. Old pluriparous mares with chronic endometritis tend to have deep pulses that can only be palpated with heavy pressure. This corresponds to an interior deficiency pattern. Young, maiden mares with endometritis tend to have superficial strong pulses that can be easily identified. Body heat is a subjective assessment, but very helpful when there is a clear "hot" body or "cold" body. Ear temperature is very helpful when assessing body heat, with a normal ear temperature being half warm (the half closer to the body) and half cool (ear tip). After the general attitude, tongue and pulse are inspected, specific acupoints on the meridians are palpated or "scanned". This procedure is performed with a blunt needle cap gently pressing the specific acupoints on meridians to identify trigger points. Acupoints that show irritation when scanned correspond to either external ailments or internal disease.

After the body is scanned, and tongue, pulse, and temperature assessed, the name of the disease, often the major complaint, such as colic, diarrhea, cough, or lameness is determined. Then the pattern of disease is identified. This is the most difficult step for those trained in Western medicine and the most important. There are six systems for differentiating disease patterns. The theory of eight principles is used most commonly and is the foundation of TCVM diagnosis. It determines if the disease is an external or an internal problem, whether the pattern of disease is hot or cold or is in excess or deficiency. The pattern can then be categorized as being either Yin or Yang. Exterior, heat and excess belong to Yang while interior, cold and deficiency belong in Yin. From the perspective of TCVM, the exterior refers to the skin, hair, and muscles. The interior refers to the Zang-fu organs. The Zang-Fu diagnostic system, another method for disease pattern identification, identifies internal medicine problems. It determines which of the Zang-fu organs has either a deficiency-excess or heat-cold pattern. The latter system combined with the eight principles is used to diagnose reproductive problems.

There are four basic forms of energy that the TCVM practitioner looks for imbalance. Diagnosing the imbalance is the key to the treatment pattern of needles and points used. The four areas of imbalance are Qi, Yin, Yang, and blood. Qi is the word for energy in TCVM, referring to the energy of each body. Yin and Yang are the two halves of Qi. The blood in the body is symbolically the river of fluid that carries the Qi. The practitioner looks for either excess or deficiency of these elements, with excess being more common in younger animals, and deficiency more common in older animals. We can have excess in heat, dry, cool, and damp, and usually deficiency in the areas of energy discussed above.

Qi is our energy, and the holding force in our bodies. Typical Qi deficient tongue and pulse patterns would be a pale, swollen or fat tongue, usually wet, and pulses weaker on the right side of the body. Basic signs of Qi deficiency are lack of stamina or energy, or of the body not "holding" either an organ or fluids – urinary incontinence, diarrhea, uterine prolapse.

Yin is our feminine energy, akin to our physiological air-conditoner, and is most active in the evening. Male or female, each body has Yin. Yin deficient patterns are diagnosed with a red or warm colored tongue, hot body, and pulses weaker on the left side. Cushing's disease is often a liver Yin deficiency (although it can have other patterns of imbalance). Human menopause is often a kidney Yin deficiency, with hot flashes (broken air conditioner) and night sweats (worse at night when the Yin is most active).

Yang is our masculine energy, of which each body should have also regardless of gender. Yang is our heater, most active during the day, and Yang deficiency is diagnosed with a very pale tongue, and pulses weaker on the right, with the addition of a cold body/ears. The key to Yang diagnosis is that it looks much like Qi deficiency but it has the added factor of cold – ears and body cold.

Blood in TCVM language is the river of fluid that carries the Qi through the body. Blood deficiency is diagnosed with a dry, dull looking tongue which can be grey or pale in color, and pulses weaker on the left. It is not unusual for Yin and blood deficiency to be seen together, as the heat from Yin deficiency can dry up the river of fluid. Both have pulses weaker on the left. If the river of blood dries up, our Qi cannot flow as easily. Blood is also the nourishment to all our tissues, most evident through the skin. Poor flaky skin can be blood deficiency or Yin deficiency. Often with our processed diets, blood deficiency can have its roots in micro-damage to the intestinal tract causing lack of nutrient absorption.

In summary, tongue, pulse, body temperature, and sensitivity to acupoints combine to make up the basic TCVM examination, and diagnosis is made based on these findings combined with history. The simultaneous simplicity and complexity of TCVM can be very frustrating for any veterinarian because these concepts sound quite ridiculous and nonsensical to the scientific mind. There have been many medical studies in the human field performed in an attempt to help scientifically understand how acupuncture works, and to help examine the significance of the energetic model of body systems for allopathic medicine.

Treatment

Treatment strategy is based on pattern differentiation. Tonification is used for a deficiency pattern, and sedation is used for an excess pattern. Warming acupuncture techniques such as moxibustion or hot herbs are used for a cold pattern, while cooling acupuncture techniques are used for a hot pattern. Certain acupoints have cooling effects whereas others have heating effects. Studies in humans indicate that correctly diagnosing the traditional Chinese medicine pattern is the most important parameter for successful outcome in treating infertility problems in women. For veterinarians trained in Western medicine identifying TCVM patterns for reproductive problems is difficult because terminology is unfamiliar as is evaluation methods. If the pattern diagnosed is incorrect, inappropriate acupoints may be stimulated resulting in a poor response.

Traditional Chinese Veterinary Medicine is directed at balancing the body so it is paramount that a thorough physical and acupuncture examination be performed to determine the disease pattern. Nutritional and environmental factors need to be discussed and questions on type of stabling, time outdoors, paddock space, traveling and competitions need to be addressed. Events occurring prior to presentation can contribute to the imbalance. Pain interferes with reproductive function and has been shown to adversely affect hormonal profiles in women. Mares that experience a dystocia, are anesthetized and hung by their hind legs, mares that raced on synthetic tracks or mares with front foot pain may exhibit irritation when acupoints overlying the sacrum, loin or hips are palpated. These mares may accumulate intra-uterine fluid because drainage is impeded due to lack of movement. When the author identifies significant musculo-skeletal, foot or dental problems in a subfertile mare, she involves a veterinarian trained in chiropractic techniques, a veterinary dentist or podiatrist to assist in alleviating the pain.

This author combines Western medicine with TCVM and has the most acupuncture experience treating chronically infertile mares with intra-uterine fluid accumulations, lymphatic lacunae and angiosis. Mares with acute bacterial endometritis are treated with uterine lavage, intra-uterine antibiotics and

oxytocin and rarely is acupuncture used in this group. Mares designated for acupuncture treatment have both a reproductive examination consisting of a thorough history, an ultrasonographic examination of the reproductive tract, a vaginal examination, uterine culture and cytology and a TCVM examination consisting of evaluation of the tongue, pulse and scanning of acupoints over the body to identify painful trigger points. Acupoints chosen for treatment typically include those that are painful on palpation, classic reproductive acupoints and acupoints for musculo-skeletal problems , if the mare exhibits lameness or pain. Treatment selection routinely includes placement of a dry needle first in Bai-Hui. The point is located at the top of the croup on the midline and following needle insertion sedation commonly occurs in the animal making it easy to place remaining needles. Needles are placed bilaterally into paired acupoints over the back and at least three bilateral pairs (i.e., BL 23 right –BL 23 left; Shen-shu right-Shen shu left) are stimulated by electro-acupuncture at a frequency of 20 Hz for five to ten minutes. The frequency is then increased to 100 to 120 Hz for ten to 15 minutes. Some mares do not tolerate this level of stimulation so the electrical current needs to be decreased to a tolerable level. Electro-acupuncture is routinely used by the author for infertility problems in mares as it has been shown to be more beneficial than dry-needles in the release of endorphins, epinephrine, catecholoestrogens and growth factors from the hypothalamic-pituitary axis in rats and women.[2] Mares treated around breeding for intra-uterine fluid retention, lymphatic lacunae or angiosis typically receive two treatments at 48 to 72 h internals before breeding and then are treated twice at 48 h intervals after ovulation but before day five of diestrus. Clinical impression is that uterine tone improves, uterine edema and fluid accumulation decreases after three to five acupuncture treatments. This protocol was developed by veterinary acupuncturists in central Kentucky.

"Cookbook" points for infertility

Correctly identifying the disease pattern is paramount for choosing the appropriate acupoints. Acupuncture "cookbooks" are available. These are lists of prescriptions of the more common acupuncture points used for the more common diseases. But, similar to Western medicine where drugs may be given without a proper diagnosis, "cookbook acupuncture" can give mediocre or disappointing results if an accurate Chinese diagnosis is not made.

The kidney and liver meridians are the primary meridians controlling reproduction. In TCVM, the kidney meridian is responsible for conception by controlling the ovaries. The liver meridian controls the uterus and cervix. Other meridians involved in reproductive function include spleen, triple heater and conception meridians (Ren channel). Many veterinarians trained in acupuncture put mares on specific herb formulations for infertility. This author has no experience with herbs, and herbal therapy must be prescribed very carefully by an experienced, trained herbalist.

Ovarian abnormalities and endometritis can be divided into six TCVM patterns: kidney Qi deficiency, liver-kidney Yin deficiency, spleen-kidney Qi deficiency, Qi-blood stagnation, liver Qi stagnation and phlegm-damp obstruction. The last three patterns are patterns of excess. Kidney Qi deficiency and liver-kidney Yin deficiency are associated with abnormalities of the ovaries and chronic endometritis. Many of the recommended acupoints are used for each condition.

For *kidney Qi deficiency*, the tongue is pale, wet with a thin white coating and the pulse is deep and weak, being more evident on the right. Clinical signs include anestrus, anovulatory follicles silent estrus and poor body condition. Points used for kidney Qi deficiency include Bai-Hui, Yan-Chi (traditional acupoints located midway between the tuber coxae and Shen-Peng; point is specific for ovarian conditions), Shen-shu, BL-23, GV-1, GV-3 and GV-4; EA bilateral Yan-Chi, Shen-shu, BL 23.

Mares with *liver-kidney Yin deficiency* have a light red tongue, dry mouth, dry hair coat, dry flaking hooves and deep pulses that are weaker on the left. Ovaries may be inactive with irregular follicular growth. Endometritis is chronic. Recommended therapy: EA bilateral acupoints: Yan-Chi, Shen-shu, Shen-Peng, and BI-23; EA at 20 Hz for ten to 15 minutes followed by 80-120 Hz for another ten to 15 minutes. Other points to consider include: Shen-jiao, LIV-1, SP-6, Sp-9, Sp-1, BL-20. It is very difficult to insert needles in acupoints SP-6 and SP-9 in Thoroughbred mares without getting kicked as they are located on the inner thighs. The author has used these points in quiet mares of other breeds.

Spleen-kidney Qi deficiency is observed in older broodmares with chronic uterine inflammation. The tongue is pale, swollen and wet and the pulse is deep and weak. The author observes this condition most commonly in older, pluriparous mares that accumulate fluid after natural mating. Recommended acupoints: BL-20, ST-36, Bl-19, BL-23, GV-3, GV-4, Kid-1, SP-6, SP-9, BL-20, BL-21, Shen-shu, Shen-Peng, Shen Jiao.

Mares with *Qi/blood stagnation* have a purple tongue and wiry pulse. Endometritis is acute to sub-acute, anovulatory follicles and irregular estrous cycles may be present. Recommended therapy: EA bilateral Shen-shu, BL-18, BL-19.

Liver Qi stagnation is seen in nervous, high strung Thoroughbred mares. The tongue is purple or red, the pulse is taut and string-like in consistency. Estrous cycles may be abnormal in length. Recommended therapy: EA bilateral acupoints Yan-chi, Shen-shu, Bl-18, BL-19, Shen-Peng at 20 Hz for ten to15 minutes followed by a frequency of 80-120 Hz for ten to 15 minutes.

Phelgm-damp is most common in obese, old, Warmblood mares with intra-uterine fluid. The tongue is pale, thick with a greasy coating. The pulse is slippery. Recommended therapy: EA Bai-hui to GV-1, Yan-chi (bilateral), Feng-long (ST 40; bilateral) and BL-19 (bilateral).

Concluding remarks

There is minimal scientific evidence that acupuncture improves reproductive function in the mare. If acupuncture is to be respected as a treatment modality it is imperative that controlled studies be designed to evaluate its effects on plasma hormones, uterine contractility and pregnancy outcome. Consistent protocols in regards to number and frequency of treatments, timing of treatments around breeding and early pregnancy need to be developed. Studies need to be conducted on animals with abnormalities as performing acupuncture studies on "balanced", healthy animals may not reveal any valuable information. A proper diagnosis is paramount to success, however, learning how to evaluate and diagnose an animal's condition using TCVM can be difficult for those trained in Western medicine. Although a "cookbook" of acupoints was presented in this text, each animal needs to be thoroughly evaluated as disease patterns will differ between individuals. Using the same protocol for a group of mares will likely produce poor results. Veterinarians interested in pursuing acupuncture for reproductive problems need to take a basic course from one of the schools in North America. This provides basic knowledge and should then be followed with working with an experienced acupuncturist for a minimum of 40 to 60 hours to improve one's ability to properly diagnose specific abnormalities. Maintaining detailed records on each case and reviewing them with other veterinary acupuncturists is helpful in the learning process.

References

1. Xie H, Preast V: Xie's veterinary acupuncture. Ames (IA): Blackwell Publishing; 2007. p. xi-xiii.
2. Kenney JD: Acupuncture and pain management. Proc Annu Conv Am Assoc Equine Pract 2011; p. 121-137.
3. Cho ZH, Hwang SC, Wong EK, et al: Neural substrates, experimental evidences and functional hypothesis of acupuncture mechanisms. Acta Neurol Scand 2006;113:370-377.
4. Helms JM: Acupuncture energetics: a clinical approach for physicians. Berkely: Medical Acupuncture Publishers; 1995.
5. Steiss J: Neurophysiological basis of acupuncture. In: Schoen AM, editor. Veterinary acupuncture. 2nd ed. Philadelphia: Mosby; 2001. p. 27-46.
6. Lin JG, Chen WL: Acupuncture analgesia: a review of its mechanism of actions. Am J Chin Med 2008;36:635-645.
7. Mayor DF: Electroacupuncture: a practical manual and resource. Philadelphia: Churchill-Livingston Elsevier; 2007. p. 59-93.
8. Lin YP, Peng Y, Yi SX et al: Effect of different frequency electroacupuncture on the expression of substance P and β-endorphin in the hypothalamus in rats with gastric distension-induced pain. Zhen Ci Yan Jiu 2009;34:252-257.
9. Son YS, Park HJ, Kwon OB, et al: Antipyretic effects of acupuncture on the lipopolysaccharide-induce fever and expression of interleukin-6 and interleukin-1 beta mRNAs in the hypothalamus of rats. Neurosci Lett 2002;319:45-48.
10. Skarda RT, Tejwani GA, Muir WW: Cutaneous analgesia, hemodynamic and respiratory effects and β-endorpin concentration in spinal fluid and plasma of horses after acupuncture and electroacupunture. Am J Vet Res 2002;63:1435-1442.

11. Malven PV, Bossut DFB, Diekman Ma, et al: Effect of naloxone and acupuncture treatment on plasma concentrations of luteinizing hormone in sheep. J Endocrinol 1984;101:76-80.
12. Xie H, Liu H: Equine traditional chinese medical diagnosis. In: Schoen AM, editor. Veterinary acupuncture. 2nd ed. Philadelphia: Mosby; 2001. p. 503-514.

The use of prostaglandin $F_{2\alpha}$ (PGF) for controlling the mare's estrous cycle

Carlos R.F. Pinto
Department of Veterinary Clinical Sciences, College of Veterinary Medicine, The Ohio State University, Columbus, OH

Introduction

Prostaglandins belong to a group of modified long-chain fatty acids containing 20 carbons called eicosanoids. The cyclooxygenase pathway uses prostaglandin synthases to convert arachidonic acid into prostaglandins. Arachidonic acid is available through the hydrolysis of phospholipids present in the cell membrane. The breakdown of membrane phopholipids is catalyzed by the enzyme phospholipase A. Two isoforms of prostaglandin synthase exist: a constitutive (cyclooxygenase-1) and an inducible (cyclooxygenase-2) isoforms. Systemic administration of prostaglandins (mainly PGF in animals) is associated with side effects affecting the central nervous system (incoordination, stupor and ataxia) and the vascular system (contraction of smooth muscle of organs such as the stomach, intestines and urinary bladder).

The use of injectable preparations of prostaglandin $F_{2\alpha}$ (PGF) has revolutionized the breeding management of horses and cattle since its identification as the main luteolytic hormone. Pharmacokinetics of PGF following intravenous administration of 5 mg per mare (large mixed breeds of large ponies) has been recently described as follows: apparent plasma clearance 3.3 ± 0.5 l/h/kg, distribution half-life of 94.2 ± 15.9 s, elimination half-life of 25.9 ± 5.0 min, and maximum plasma PGF concentration of 249.1 ± 36.8 ng/ml.[1] The original studies pointed out that mares seemed more sensitive to exogenous PGF than cows. Indeed, an *in vitro* study has shown the affinity of equine luteal cell membrane preparations for PGF to be approximately 10 times greater than that for bovine luteal cell membrane preparations.[2] The relatively high affinity of mare corpus luteum (CL) to binding of PGF along with the relatively slow metabolic clearance documented in mares account for the greater sensitivity of mare CL to the luteolytic effect of PGF when compared to other domestic species.

The luteal phase of the equine estrous cycle can be reliably shortened by the administration of PGF allowing mares to return to estrus at a relatively predictable time (on average two to five days after PGF administration). In horses, a single treatment with PGF will induce complete luteolysis if administered at least five to six days after ovulation. This fact led to the prevailing assumption that the CL is not responsive to PGF luteolytic effects before it is at least five days old, despite the fact that some initial studies reported that some mares were responsive to luteolytic effect of PGF administration when treated on Day 3 after ovulation. In the USA, the natural analogue dinoprost tromethamine[a] is the only FDA-approved PGF for use in horses, although equine practitioners commonly use the synthetic analogue cloprostenol in breeding management mostly owing to the longer half-life than its natural analogue. A review of the effects of PGF on luteal function and characteristics of the subsequent induced estrus and ovulation will be presented in the subsequent sections. Use of PGF as an abortifacient and ecbolic actions of PGF for use during breeding management will not be discussed in this manuscript.

Effects of PGF administration on the mare's reproductive cycle

Natural luteolysis begins approximately 14 days after ovulation in mares. In the decade of 1970s, several studies investigated the effects of PGF treatment on blood progesterone concentration profile and effects of the length of diestrus and interovulatory intervals.[3,4,5] Most of these studies were based on examinations of serial blood samples taken before and after treatment with PGF or based on the recording of the length of interovulatory intervals in treated and control mares. Studies on subsequent PGF-induced estrus, follicular dynamics and ovulation were based mainly on findings of serial reproductive examinations utilizing palpation per rectum. More recently, a significant wealth of information on the characteristics of luteal development and regression, follicle growth and ovulation following PGF-induced luteolysis became available with the advent of transrectal ultrasonography. The information gained with

ultrasonography studies on mare reproduction contributed to the understanding of PGF actions on the mare's reproductive cycle and tract.[6,7]

Soon after PGF was shown to be the uterine luteolysin in cattle, sheep and in rats, Douglas and Ginther published in 1972 convincing evidence that exogenous (subcutaneous or intramuscular) or intrauterine administration of PGF had also luteolytic effects in mares.[4] Since then, PGF and its synthetic analogues have been widely used in breeding farms that require intensive management of broodmares and stallions and mares.[7] In the original study by Douglas and Ginther (1972) mares received PGF treatment on day six after detection of ovulation because it had been previously shown that intrauterine infusions of saline solution performed six days after ovulation would shorten the mare's estrous cycle as denoted by an interruption of luteal activity that terminated diestrus and by a shortened interovulatory interval.[4] In that study, all mares treated with 1.25, 2.5, 5.0 or 10.0 mg of PGF had shorter diestrus and shorter interovulatory intervals than control mares (not treated with PGF). Following that report, several other studies confirmed that PGF treatments not only shorten diestrus but also interovulatory intervals. Despite the fact some mares may undergo complete luteolysis when treated on Day 3 after ovulation, maximal response to one single bolus injection is expected when at least five days have elapsed from ovulation. Anecdotally, some equine practitioners report that, whenever the day of ovulation is unknown, daily treatments of PGF are prescribed until treated mares show signs of behavioral estrus.

Luteolytic doses of PGF preparations

Dinoprost tromethamine

For PGF tromethamine salt preparations (PGF tham salt), 1.34 mg of the salt equals 1 mg of free acid PGF. Douglas and Ginther (1972) reported that doses of 1.25, 2.5, 5.0 and 10.0 mg of PGF were all found to shorten the luteal phase of the estrous cycle. Mares in all treatment groups were found in estrus three to four days after treatment. In horse mares, a single bolus dose of 1.25 mg of dinoprost tromethamine per horse mare (~ 2.8 ug/kg for an average 450 kg mare) when administered between days six and 12 after ovulation has been shown to be luteolytic and induce normal ovulatory estrus periods, which in turn were followed by normal luteal function (diestrus).[8] Even doses as low as 0.5 mg per mare (~ 1.1ug/kg) has been shown to affect luteal function; however, complete luteolysis (21/21 mares) was only achieved when mares were treated twice 24 hours apart.[9] In that study, this low dose did not induced common side effects (sweating, colicky behavior) generally associated with PGF treatment. Most commercial preparations of dinoprost tromethamine, however, recommend a single intramuscular or subcutaneous bolus administration of 5 to 10 mg per mare (~ 11.1 to 22.2 ug/kg).

Cloprostenol

In contrarst to several other countries, cloprostenol formulations are not FDA-approved for use in horses in the USA. Nevertheless, cloprostenol is widely used in the USA by equine practitioners mainly because of its longer half-life and association with lesser side effects than dinoprost tromethamine. Cloprostenol is available as two optically active isomers (enantiomers), d-cloprostenol and l-cloprostenol. The recommended luteolytic doses of these synthetic analogues are much lower than that recommended for the natural analogue dinoprost tromethamine. Luteolytic doses for d-cloprostenol are further lower than that needed for d,l-cloprostenol-induced luteolysis.[10] The dosage difference between these two cloprostenol analogues is explained by the fact that only the d-enantiomer is pharmacologically active (luteolytic). Most popular preparations of cloprostenol in the USA use the racemic mixture[b] (d- and l-enantiomers) at a dose of 250 to 500 ug per mare. In one study, doses as low as 25 ug of d,l cloprostenol per mare successfully induced luteolysis.[11] In several countries, the more potent preparations using only the active d-cloprostenol enantiomer[c] are also available and labeled for use in horses. In a recent report, the bolus dose of 37.5 ug of d-cloprostenol[c] was found to induce complete luteolysis similar to mares receiving 250 ug of a d,l-cloprostenol preparation.[12] The recommended labeled doses for d-cloprostenol and d,l cloprostenol are 37.5 ug per mare (0.5 mL injection volume) and 250 ug (1 mL injection volume), respectively, administered subcutaneously or intramuscularly.

Luteolytic effects of PGF and stage of the estrous cycle

The results presented in the early studies in the 1970's provided the basis for the assumption that PGF formulations would not induce luteolysis or affect CL function if administered before Day 5 or 6 post-ovulation. Interestingly, some authors reported that some mares actually responded to PGF-induced luteolysis when treated on Day 3 post-ovulation;[3] however, the notion that the early CL was not responsive to PGF administration remained ingrained in the scientific and veterinary professional community. In 1974, Thompson and Witherspoon briefly reported another phenomenon that only recently has gained attention: the ability of PGF to induce partial luteolysis followed by resurgence in CL function that is characterized by a transient increase in concentrations of blood progesterone.[13] In that study, two mares receiving a relatively low dose of a synthetic PGF analogue nine days after ovulation began to experience a decrease in concentrations of plasma progesterone at 12 hours after PGF treatment followed by a resurgence in progesterone concentrations at 48 hours after treatment; progesterone concentrations then remained at 30% to 50% of that before PGF treatment. More recently, 32 years from that initial report, Bergfelt et al (2006) compared the pattern of luteolysis following PGF treatment as a single bolus injection on Day 3 after ovulation with that of mares treated on Day 10.[14] In the Day 3 group, 75% (12/16) of mares experienced CL resurgence. Among those, six mares experiencing "minor" progesterone resurgence had similar treatment-to-ovulation intervals to control mares. In summary, the phenomenon of CL resurgence following PGF treatment reflects a condition by which the CL to undergo partial luteolysis, as denoted by decreasing concentrations of blood progesterone followed by resurgence of the CL function, denoted by a modest but significant transient increase in progesterone concentrations. Partial luteolysis followed by CL resurgence may occur following administration of sub-luteolytic boluses doses of PGF during mid diestrus,[13,15,16] or following administration of single injections at Day 3 after ovulation.[14]

Effects of exogenous PGF on steroid and gonadotropin secretion

Administration of PGF in mares with a functional CL >5 days after ovulation is followed by functional luteolysis (significant decrease in progesterone) 24 hours after treatment that is, however, preceded by an immediate, transient rise in progesterone shortly after PGF treatment. Noden et al (1978) reported that functional luteolysis was preceded by a transient increase in progesterone, estradiol and luteinizing hormone (LH) at 10, 30 and 60 min after PGF treatment of diestrual mares.[17] In a more recent study by Ginther et al (2009), administration of a single luteolytic intravenous bolus of PGF resulted in an immediate increase in circulating progesterone concentrations within 10 minutes following the bolus injection accompanied by an increase in concentrations of follicle stimulating hormone (FSH), LH, and cortisol.[18] Conversely, mares infused with PGF for two hours, mimicking a natural pulse of endogenous PGF action, did not show increases in the same hormones; however, both treatments, bolus injection and infusion, resulted in similar luteolytic effects. These effects on steroids and gonadotropin secretion associated with supraphysiologic doses of PGF may partially explain the results of one study that found that mares treated in estrus with a synthetic PGF, fenprostalene, had shorter estrus-to-ovulation intervals than control mares.[19]

PGF treatment and antiluteogenesis

Recently, it has been reported that luteolysis or prevention of luteal formation may be accomplished with PGF administration beginning as early as the day ovulation is detected. This effect is dependent on the dose and frequency of PGF treatments. Based on the fact that the early developing CL <5 days is actually responsive to luteolytic effects of PGF, a series of experiments conducted in our laboratory produced data that support the hypothesis that the early developing CL is indeed responsive to exogenous PGF as early as within the first 24 hours from ovulation.[20,21] Because of this early luteolytic responsiveness to PGF administration before the CL is fully functional, we named this phenomenon as (PGF-induced) *antiluteogenesis*. Mares treated once or twice daily for three days with 2.5 or 10 mg of

dinoprost failed to show a significant rise in concentrations of plasma progesterone during the treatment period. Approximately 60% of mares treated twice daily for three days with 10 mg of PGF experienced complete luteolysis where all mares receiving once daily 2.5 mg of PGF for three days showed CL resurgence. Therefore, the antiluteogenesis effect of PGF is dependent on the dose and frequency of PGF treatments.

Clinical applications of PGF in broodmare management

Use of PGF to induce luteolysis and return to estrus

Termination of the luteal phase ("short-cycling") with exogenous PGF may be attempted for planned breeding of a single mare or as an approach to synchronize estrus and ovulation in a group of mares. If reproductive examinations with palpation per rectum and transrectal ultrasonography are available, the predictability of onset of estrus and ovulation increases. Prediction of the next ovulation in the PGF-induced estrus is not predictable as it is the return to estrus. For example, it has been shown that the diameter of follicles present in the ovaries at the time of PGF treatment may influence when the mare would ovulate. When a relatively large follicle (35 mm or greater) is present at the time of PGF administration, the onset of estrus and ovulation will depend on the follicular status (growing phase vs. undergoing atresia). Accordingly, mares with follicles approaching the diameter of preovulatory follicles may come in estrus and ovulate within two to five days following PGF treatment, whereas the mean interval from treatment to ovulation in mares during mid diestrus and with follicles <25 mm may vary from seven to 12 days from treatment. For example, in some extreme instances, mares will ovulate in two to three days; mares ovulating within 48 hours from PGF treatment often show no signs of behavioral estrus. Conversely, larges follicle present the time of PGF treatment may be already undergoing atresia will slowly regress and the mare may not ovulate until ten to 14 days after the treatment. In most cases, however, mares will come into estrus and the large follicle at the time of PGF treatment will continue to grow and ovulate within four to six days after PGF treatment.

Obviously the prediction of PGF-induced estrual events requires that treated mares have an active corpus luteum at the time of administration. If reproductive examination is not available, horse owners may be instructed to administer a single dose of PGF five days after the mare ceases behavioral signs of estrus, or alternatively, if teasing is not feasible, daily administration of a single PGF treatment may be prescribed until the mare shows signs of estrus or a reproductive examination by a veterinarian becomes available. Another alternative if veterinary assistance or teasing information were not available, would be to recommend administration of a single dose of PGF at any given day and to repeat it in five days if the mare is not observed in estrus.

Use of PGF in postpartum mares

Several factors associated with complications during foaling could compromise the fertility of the mare's foal heat. For most mares experiencing dystocia or retention of the fetal membranes, it may be prudent to not breed on the first estrus following parturition (foal heat). In this scenario, instead having horse owners waiting for mares to come into their second postpartum estrus ("thirty day heat"), one strategy would be to treat mares with PGF approximately five to seven days after ovulation in the foal heat.

Use of PGF mares with prolonged luteal phases

Occasionally, mares may experience prolonged diestrus periods owing to the presence of a persistent CL. Persistent CLs may occur in mares that failed to express their endogenous luteolytic mechanism (rare), or more commonly occur in mares that experience early embryonic loss after maternal recognition of pregnancy takes place. In general, prolonged diestrus is often associated with another unique phenomenon of the mare's estrous cycle, the diestrus ovulation. Prolonged diestrus is diagnosed as a diestrus period lasting more than 16 days after ovulation. A single dose of PGF should induce mares to return to estrus.

Use of PGF in estrus synchronization

One of the most basic methods to attempt estrus synchronization is to treat mares with PGF and repeat the treatment approximately two weeks from the first injection. If teasing is available, mares can then be teased every other day beginning two days after PGF treatment. The efficacy of the use of PGF in estrus synchronization programs is greatly enhanced with the concomitant use of progestagens and estrogens.

Non-reproductive effects associated with PGF administration

In general, prostaglandins have significant effects on vascular and non-vascular smooth muscle, central nervous systems and carbohydrate and lipid metabolism.[22] The administration of exogenous PGF is relatively safe and doses 20-40 times greater than the therapeutic dose (typically 5 to 10 mg of dinoprost) do not elicit toxic effects.[23] Even doses up to 800 mg were not fatal to mares despite being associated with intense side effects such as recumbency; in that study severe side effects subsided by four to five hours after PGF overdose treatment. This increased sensitivity is also reflected by the appearance of side effects following administration of a conventional luteolytic dose in mares in 20 to 40% of mares treated with PGF: sweating, restless behavior, diarrhea or even colic-like signs are commonly observed in mares but not in cattle. One of the most common side effects is pronounced sweating seen within minutes following PGF administration. The results of most research studies indicate that equine sweating occurs by stimulation of adrenoreceptors on the sweat gland cells.[24] Adrenaline-induced sweating is primarily mediated by β2 adrenoreceptors. Horses given PGF intramuscularly sweat but do not shiver, although shivering occurs in horses treated with adrenaline; this may explain why rectal temperature significantly decreases in horses after PGF administration.[23] Because concentrations of plasma adrenaline and noradrenaline become elevated after administration of PGF it has been accepted that PGF-related sweating is associated with release of adrenaline from the adrenal medulla. Some mares may also experience abdominal discomfort resembling colic-like symptoms. Abdominal discomfort is a result of hypergastromotility. Occasionally, some mares also show locomotor incoordination and ataxia. These side effects typically subside within 20 to 30 minutes after PGF treatment. The appearance and duration of these aforementioned side effects seem to vary among mares. It is important to note that these side effects are dose dependent and typically subside within the first hour following PGF treatment. Irvine et al (2002) reported that the administration of two low doses of PGF 24 hours apart did not elicit any appreciable side effects, including elevation in heart rate.[9]

Conclusions

Manipulation of the mare's estrous cycle with PGF is an important strategy in the breeding of mares. The CL is sensitive to PGF treatment throughout the whole estrous cycle. A single bolus injection of PGF can reliably induce luteolysis when administered in mares with a CL >5 days. Serial injections of PGF for several days beginning (q 12 or q 24 h) as early as within 24 hours from ovulation will prevent CL formation (antiluteogenesis) as evidenced by the absence of a rise in progesterone. Not only diestrus is shortened in mares treated with PGF but interovulatory intervals are also reduced in relation to normal, untreated cycles. Estrus and ovulation occurring after PGF treatments are normal and the inherent fertility of mares treated is not affected.

Footnotes:

a. Dinoprost tromethamine; Lutalyse®; Pfizer Animal Health, Kalamazoo, MI
b. d,l cloprostenol sodium; Estrumate®; Merck Animal Health, Union, NJ
c. d, cloprostenol; Genestran®; FORTE Healthcare Limited; Naul, Dublin, Rep of Ireland

References

1. Shrestha HK, Beg MA, Burnette RR, et al: Plasma clearance and half-life of prostaglandin F2alpha: a comparison between mares and heifers. Biol Reprod 2012;87:1-6.

2. Kimball FA, Wyngarden LJ: Prostaglandin F2alpha specific binding in equine corpora lutea. Prostaglandins 1977;13:553-564.
3. Douglas RH, Ginther O: Effect of prostaglandin F2alpha on length of diestrus in mares. Prostaglandins 1972;2:265-268.
4. Oxender WD, Noden PA, Louis TM, et al. A review of prostaglandin F2alpha for ovulation control in cows and mares. Am J Vet Res 1974;35:997-1001.
5. Oxender WD, Noden PA, Hafs HD: Oestrus, ovulation and plasma hormones after prostaglandin F2alpha in mares. J Reprod Fertil Suppl 1975;23:251-255.
6. Gastal EL, Bergfelt DR, Nogueira GP, et al: Role of luteinizing hormone in follicle deviation based on manipulating progesterone concentrations in mares. Biol Reprod 1999;61:1492-1498.
7. Goretti RG, Araújo RR, Filho AN, et al: Effects of timing of induced luteolysis in embryo donor mares on reproductive performance and pregnancy rate in recipient mares. Theriogenology 2011;75:1170-1174.
8. Barker C, Echeverria, Davis M, et al: Effects of different doses of $PGF_{2\alpha}$ on luteal function and on the subsequent estrous cycle. Anim Reprod Sci 2006;94:207-209.
9. Irvine CH, McKeough VL, Turner JE et al: Effectiveness of a two-dose regimen of prostaglandin administration in inducing luteolysis without adverse side effects in mares. Equine Vet J 2002;34:191-194.
10. Beretta C, Cavalli M: A sheer pharmacologic approach to compare the contractile effects of PGF2alpha, DL-cloprostenol and D-cloprostenol on isolated uterine, tracheal, ileal and arterial smooth muscle preparations. Theriogenology 2004;62:837-846.
11. Nie GJ, Goodin AN, Braden TD,et al: Luteal and clinical response following administration of dinoprost tromethamine or cloprostenol at standard intramuscular sites or at the lumbosacral acupuncture point in mares. Am J Vet Res 2001;62:1285-1289.
12. Cuervo-Arango J, Newcombe JR: Relationship between dose of cloprostenol and age of corpus luteum on the luteolytic response of early dioestrous mares: a field study. Reprod Domest Anim 2012;47:660-665.
13. Thompson FN, Witherspoon DM: Induction of luteolysis in the mare with a prostaglandin analogue. Theriogenology 1974;2:115-119.
14. Bergfelt DR, Pierson RA, Ginther OJ: Regression and resurgence of the CL following PGF2alpha treatment 3 days after ovulation in mares. Theriogenology 2006;65:1605-1619.
15. Witherspoon DM, Lamond DR, Thompson FN, et al: Efficacy of a prostaglandin analogue in reproduction in the cycling mare. Theriogenology 1975;3:21-30.
16. Kiefer BL, Roser JF, Evans JW, et al: Progesterone patterns observed with multiple injections of a PGF-2 alpha analogue in the cyclic mare. J Reprod Fertil Suppl 1979;27:237-244.
17. Noden PA, Oxender WD, Hafs HD. Early changes in serum progesterone, estradiol and LH during prostaglandin F2alpha-induced luteolysis in mares. J Anim Sci 1978;47:666-671.
18. Ginther OJ, Siddiqui MA, Beg MA: Physiologic and nonphysiologic effects of exogenous prostaglandin F2alpha on reproductive hormones in mares. Theriogenology 2009;72:417-424
19. Savage NC, Liptrap RM: Induction of ovulation in cyclic mares by administration of a synthetic prostaglandin, fenprostalene, during oestrus. J Reprod Fertil Suppl 1987;35:239-243.
20. Rubio C, Pinto CR, Holland BE, et al: Anti-luteogenic and luteolytic effects of $PGF_{2\alpha}$ during the post-ovulatory period in mares. Theriogenology 2008;70:58.
21. Holland BE; Pinto CRF: Luteal function and ovulation in mares treated with $PGF_{2\alpha}$ during early and mid-diestrus. Reprod Domest Anim 2008;43:111.
22. Smith ER, Mason MM: Toxicology of the prostaglandins. Prostaglandins 1974;7:247-268.
23. Goyings LS, Lauderdale JW, McConnell RF: Pharmacologic and toxicologic study of prostaglandin F2alpha in mares. Am J Vet Res 1977;38:1445-1452.
24. Jenkinson DM, Elder HY, Bovell DL: Equine sweating and anhidrosis Part 1--equine sweating. Vet Dermatol 2006;17:361-392.

Update on placentitis – what has been accomplished in the last 3 years?

C. Scott Bailey
College of Veterinary Medicine, North Carolina State University, Raleigh NC

Abstract

Bacterial placentitis is among the leading causes of abortion in the mare. Further, despite intense research and clinical focus, diagnosis for most forms of placentitis remains difficult and clinical treatment success remains limited. As clinical signs are non-specific and may occur late in the course of disease, screening tests have been developed, including ultrasonographic evaluation of the combined thickness of the uterus and placenta, hormonal assays and measurement of acute phase proteins. Mares diagnosed with placentitis are generally treated with a combined regimen including an antibiotic, such as trimethoprim sulfamethoxazole, anti-inflammatory therapy, such as flunixin meglumine or pentoxifylline, and altrenogest. However, despite a large body of research, clinical outcomes remain poor. Key challenges that must be addressed to improve clinical outcomes for mares with placentitis are 1) the development of affordable, sensitive screening tests; 2) identification of effective anti-inflammatory therapy to prevent abortion and fetal compromise; and 3) identification of more effective antibiotic therapy.

Keywords: Equine placentitis, diagnosis, trimethoprim sulfamethoxazole, NSAID, preterm delivery

Introduction

Bacterial placentitis is among the leading causes of abortion in the mare. Further, despite intense research and clinical focus, diagnosis for most forms of placentitis remains difficult and clinical treatment success remains limited.

A series of retrospective studies of equine abortion from the US and Europe document the importance of this disease, and further subcategorize placentitis into subcategories.[1-3] The most common type of placentitis is ascending bacterial placentitis, which in turn is most commonly caused by the organisms *Streptococcus equi* subsp. *zooepidemics* (*S. zooepidemicus*) or *Escherichia coli*. In addition to being the most common cause of equine endometritis and ascending placentitis, *S. zooepidemicus* has recently gained additional notoriety for its ability to "jump" species.[4] It has been reported as an occasional cause of mastitis in cattle and a cause of canine infectious respiratory disease and hemorrhagic streptococcal pneumonia (HSP) in dogs.[5,6] There have been numerous reports of disease in people, occasionally associated with mortality.[7]

Other economically important types of placentitis are nocardioform placentitis, leptospiral placentitis, and the twin conditions equine amnionitis and fetal loss syndrome (EAFLS) and mare reproductive loss syndrome (MRLS). While local outbreaks of each of these diseases has been reported in recent years, the current manuscript will focus on ascending placentitis.

Pathophysiology

Recent reviews by Ousey and Fowden describe the extensive work that has characterized the endocrine control of normal parturition and abortion.[8-10] Pregnancy maintenance and parturition are controlled by a complex set of hormonal interactions which serve to first prevent and then promote uterine contractions. In other species, progesterone has been demonstrated to promote "uterine quiescence" at least in part through an upregulation of 15-hydroxy prostaglandin dehydrogenase (PGDH), which inactivates prostaglandins,[11] and by preventing prostaglandin-upregulation by cortisol.[12] In the mare, this correlation is less well understood. Progesterone is virtually absent from systemic circulation in late pregnancy, and it has further been demonstrated that the uterus of pregnant mares continues to respond to either oxytocin or prostaglandin with increased muscular contractility. However, it is possible that either progesterone or one of the progestagens produced by the fetoplacental unit act in a paracrine fashion to diminish uterine contractility in pregnant mares, thus preserving normal pregnancy.[8]

Mares with experimentally-induced placentitis have been shown to have increased allantoic fluid concentrations of prostaglandin E and prostaglandin F2α. At the time of delivery, proinflammatory cytokines were substantially elevated in placental tissues of mare with intrauterine disease, compared to those that

delivered normally.[13,14] These findings correspond with work in women, non-human primates, as well as other animal models of preterm labor. Proinflammatory cytokines and prostaglandins both serve to activate the fetal hypothalamic-pitutary-adrenal axis (HPA axis).[15] The fetal adrenal produces both progestins and, once sufficiently mature, cortisol.[15,16] Fetal cortisol, in turn, enhances placental and uterine prostaglandin production, further enhancing uterine contractility and resulting in fetal delivery.[17]

In addition to its role in causing preterm delivery, recent work in non-human primates further suggests strongly that proinflammatory cytokines have direct detrimental effects on the fetal brian and neurologic system, independent of bacterial damage or anoxia.[18,19] These new findings have not been confirmed in the horse, but may represent a component of the disease-processes frequently encountered in post-natal foals born to mares with placentitis, even in the absence of detectable bacterial infection or peripartum complications.

Diagnosis

Rapid diagnosis and treatment of disease likely play a key role in veterinarians' ability to affect a positive treatment outcome. However, placentitis often does not result in obvious clinical signs prior to abortion or delivery, and systemic health parameters (temperature, pulse, respiration) or hematologic parameters (complete blood count and serum chemistry values) are generally within normal limits, even in the face of severe intrauterine disease.[20] In an experimental model of induced placentitis, mucoid, purulent or serosanguinous vulvar discharge was the most common clinical sign, however this is infrequently noticed or reported by owners and veterinarians.[21] In contrast, in the author's experience, the most common presenting complaint in naturally occurring cases of either ascending placentitis or nocardioform placentitis is precocious mammary development prior to day 300-310. The mechanism for such mammary development has not been fully elucidated, but it is presumed to correspond with increasing maternal serum progestin concentrations, as in normal parturition. This would indicate that these changes occur only after substantial fetal compromise and suggest the strong need for more sensitive diagnostic and screening tools.

Ultrasonography

Ultrasonographic evaluation of the uterus and placenta during late gestation was first described by Renaudin and Troedsson in 1997, and has since been widely accepted as an effective screening tool.[22] The mean thickness of the uteroplacental unit in nine Thoroughbred and Quarterhorse mares plus two times the standard deviation (95% confidence interval) was less than 7 mm for mares up to 270 days of gestation, less than 8 mm for mares between 271 and 300 days of gestation, less than 10 mm for mares between 301 and 330 days of gestation and less than 12 mm for mares greater than 330 days gestation. Work by da Silva and coworkers in Warmblood mares and independent work in our laboratory in pony mares recently confirmed that these same values can be applied to a wide range of mares.[23,24] In our laboratory, Doppler ultrasonography did not identify alterations in uterine blood flow in mares with experimentally-induced placentitis.[24] At this time, ultrasonographic examination of the combined thickness of the uterus and placenta represents the most sensitive and specific diagnostic and screening tool and can be recommended for valuable or high-risk patients. In one large farm where mares were routinely screened and treatment based on alterations of the CTUP, pregnancy and neonatal losses were reduced by roughly 50% (Zent W, personal communication, 2008). However, frequent monitoring of mares with no known risk factors for placentitis represents a significant expense and may not be cost-effective or feasible.

Hormone assays

Measurement of serum progestins (evaluated via the test-specific cross-reactivity with progesterone) has also been used in research and clinical settings for diagnosis of placentitis. Systemic progestagen concentrations in healthy mares are very low during late gestation and have been shown to rise in conjunction with fetal adrenal maturation after 310 days postovulation, with rapid a decline 24-48 hours prior to parturition.[25] In mares with compromised pregnancies, progestagen levels have been shown to follow one of two patterns: they may either drop precipitously before fetal demise or abortion[26,27] or they may be prematurely elevated.[26-28] Premature elevations of maternal serum progestagen concentrations are consistent

with fetal stress and premature activation of the fetal adrenal and these changes may be used as a screening or confirmatory diagnostic tool in mares considered at risk for placentitis.[27,29] Relative sensitivity of ultrasound and progestagen assays in naturally occurring disease has not been evaluated.

Biochemical and protein markers

In women, biochemical studies have demonstrated promise, with a range of markers detectable in amniotic fluid, serum and cervical mucus.[30,31] Recent work in mares with experimentally induced placentitis also found detectable differences in the inflammatory acute phase proteins serum amyloid A (SAA) and haptoglobulin between mares with placentitis and those without disease.[32] A separate, more comprehensive study compared SAA concentrations in normal mares during late gestation to mares with experimentally induced placentitis.[23] These authors found that SAA remained low until between 120 hours before and 36 hours after parturition in normal mares, at which time a substantial increase in serum concentrations was detectable until about 60 hours after parturition. Infected control mares developed elevated SAA concentrations within 48-144 hours after inoculation. However, in that study, clinical diagnosis preceded the rise in SAA by approximately 24 hours. Treatment was initiated based on clinical signs, and 6/9 treated mares did not experience a rise in SAA. These findings warrant further work in clinical cases of naturally-occurring placentitis to determine whether SAA may be used as an inexpensive, sensitive screening tool for placental function and whether it is influenced by confounding factors, such as extra-uterine disease or obesity.[33]

Rapid diagnosis and screening of high-risk mares may be best performed using a combination of techniques, such as regular biochemical or hormonal screening, combined with intermittent ultrasonographic examination. The cost of such screening represents a major barrier to early diagnosis of placental disease.

Treatment

Based on the pathophysiology of disease and clinical experience, treatment of ascending placentitis, as well as nocardioform placentitis, has relied on a regimen combining antibiotics, anti-inflammatory or immune-modulatory medication, and progestins.[34] Extensive work at the University of Florida has examined the penetration of common equine therapeutics to the allantoic fluid and to placental and fetal tissues.[35-38] Further, a clinical trial utilizing the commonly used combination of trimethoprim sulfamethoxazole (TMS), pentoxifylline (PTX) and altrenogest (ALT) in experimentally-infected mares resulted in the delivery of 83% viable foals, compared to no viable foals in the control group.[20] However, clinical experience has not been able to confirm similar positive outcomes in naturally infected mares. Furthermore, recent work in our laboratory which delayed treatment onset until CTUP was above published normal values resulted in only 40% viable foals.[24] These findings strongly suggest a need for further research to better understand the mechanistic actions of commonly used drugs at the level of the uterus, placenta and fetus. In this review, we will summarize what is known regarding each of the commonly used therapeutics.

Antibiotic drugs

The first line of defense against placentitis is antimicrobial therapy. Antibiotic agents used to treat placentitis in mares include cephalosporins, tetracyclines, sulfonamides, trimethoprim, carboxypenicillins and penicillin plus betalactamase inhibitors.[21] These drugs have good *in vitro* sensitivity against the most common organisms causing ascending placentitis, including *S. zooepidemicus* and *Escherichia coli*.[21] Work by Macpherson and co-workers has further established that gentamicin, penicillin G and TMS each achieve therapeutic concentrations within allantoic fluid.[36,37,39] Penicillins are highly effective against *S. zooepidemicus*, while gentamicin is effective against most gram- organisms.[40,41] Due to the potential for mixed infections, hospitalized patients are generally treated with a combination of standard doses of penicillin G and gentamicin.[42,43] However, the need for repeated drug administration and catheter-maintenance makes this combination impractical for prolonged therapy of patients maintained in a farm setting, and TMS is widely used for this purpose.[34] Trimethoprim sulfamethoxazole is a broad-spectrum, bacteriocidal antibiotic with good *in vitro* activity against common causative organisms of placentitis.[35,36,41] Interestingly, despite good clinical outcomes, even prolonged treatment with TMS failed to reliably clear *S. zooepidemicus* from

mares' uteri in a model of experimentally-induced mares.[20] In that study, seven of 12 mares that were experimentally infected with *S. zooepidemicus* and subsequently treated, had positive *S. zooepidemicus* growth within six hours of foaling, compared to zero of 18 normal foaling mares.[44] Work in our laboratory has subsequently confirmed these findings and also confirmed that the organisms cultured at parturition were identical to the inoculated strain based on high performance lipid chromatography (HPLC) testing, and that it was sensitive to TMS (Bailey, unpublished data). Macpherson and coworkers recently completed a study characterizing allantoic, fetal and placental concentrations of ceftiofur crystalline free acid. This work failed to detect therapeutic concentrations of ceftiofur in allantoic samples or fetal samples.[38] These findings, combined with the poor treatment outcome of natural infections, point toward a need for continued research into the efficacy of antibiotics for the treatment of equine placentitis.

Nonsteroidal anti-inflammatory drugs (NSAIDS)

Flunixin meglumine is commonly used by clinicians as a component of treatment for placentitis, and a retrospective study by Zent and coworkers suggested that a therapeutic regimen including anti-inflammatory medication such as flunixin meglumine could improve foal viability.[21] Further, in mares experimentally injected with endotoxin in early gestation (day 21-35), flunixin meglumine prevented prostaglandin synthesis and subsequent luteolysis, resulting in maintenance of pregnancy.[45] It was not used in recent clinical trials[20,24] due to the fact that it previously had not been detected in allantoic fluid of normal pregnant mares or mares with experimentally-induced placentitis after fluid collection via microdialysis.[36] Thus, it was not known whether flunixin meglumine could penetrate through the placenta and reach target locations, including fetal fluids and tissues. However, it is possible that the drug was present in allantoic fluid, but not detected due to the nature of the assay, which allowed only small molecules to be collected. Further, recent work in our laboratory utilizing an *in vitro* model of placental inflammation confirmed that flunixin meglumine effectively inhibits both prostaglandin E and prostaglandin F2α production by chorioallantoic tissue (Bailey unpublished data). The use of other NSAIDS, such as aspirin has further been suggested, but limited studies have been performed to support their use at this time.

Other anti-inflammatory drugs

Pentoxifylline is a methylxanthine derivative that is widely used in the treatment of placentitis. In experimental models of placentitis, it was found that administration of PTX and TMS tended to prolong the interval from infection to delivery compared to animals infected and not treated, and that a combination of PTX, TMS and ALT resulted in significantly prolonged intervals between infection and foaling and significantly more viable foals than no treatment.[20,35] However, its mechanism of action in the treatment of placentitis is not well understood. In models of endotoxemia, pentoxifylline was shown to have anti-inflammatory effects by inhibiting the cytokines TNF and IL-1β,[46,47] and decreasing prostaglandin F2α concentrations.[48] In other models, it further has been suggested to have vasodilatory and rheostatic effects.[49,50] Longterm administration of pentoxifylline has been confirmed to enhance uterine artery bloodflow in mares and women,[50-52] however a recent study in our laboratory did not find that short-term administration of pentoxifylline twice daily for three days could increase uterine artery bloodflow.[53] Based on the short interval between diagnosis of placental infection and abortion in untreated, experimentally infected mares,[20,24,32] the author concluded that any primary effect of pentoxifylline is most likely not mediated through a rheostatic or vasodilatory effect and would most likely be anti-inflammatory in nature. Further work is needed to establish the role of pentoxifylline in treating mares with placentitis.

Women with preterm labor are frequently administered glucocorticoids, and work in non-human primates with intrauterine infections further demonstrated that glucocorticoids significantly inhibited inflammatory mediators and uterine activity.[54,55] In mares, work by Ousey and coworkers showed that glucocorticoids therapy could enhance fetal maturation and result in birth of precociously mature viable foals from healthy mares and mares with non-placentitis gestational disease.[28,56,57] However, a small trial at the University of Mississippi found that treatment with TMS alone was as effective as dexamethasone and TMS combined.[58] It is known that cortisol and other glucocorticoids are a vital component of fetal maturation prior to term, but also that endogenous or exogenous glucocorticoids enhance placental prostaglandin production.[8]

Further work is needed to determine whether glucocorticoids therapy is efficacious and could improve fetal viability in mares with placentitis.

Tocolytic drugs

Betamimetics and other tocolytic agents, such as oxytocin antagonists and magnesium sulfate are widely used in the treatment of preterm labor in women, but remain controversial.[59,60] In mares, limited studies have investigated the safety or efficacy of tocolytic agents. Palmer and co-workers investigated the effect of clenbuterol in term mares at multiple doses and were unable to inhibit parturition with this agent.[61] At this time, only progestins are used routinely for tocolysis in cases of equine placentitis. The rationale for this therapy in preterm labor stems from the role progestins play in inhibiting formation of myometrial gap junctions, which facilitate uterine contractility. These findings were first demonstrated by Garfield and co-workers through *in vitro* studies of ovine endometrium.[62] More recent *in vitro* studies have shown that progesterone interferes with the binding of oxytocin to its receptor and inhibits prostaglandin secretion.[63] In women, clinical data clearly demonstrate that progestins are effective at preventing preterm labor,[64] and at this time, progestins are widely accepted as a key component of therapy for preterm labor in women.[65,66] Likewise, several *in vivo* trials, utilizing altrenogest in early- and mid-gestation mares, demonstrated that, a synthetic progestin could prevent abortion. In a study by Daels and co-workers, altrenogest was effective at promoting pregnancy maintenance after intravenous infusion of *Salmonella typhimurium* endotoxins to mares between days 21 and 35 of gestation.[67,68] Mares treated with 44 mg of altrenogest, daily until day 70, maintained gestation to normal term and delivered live foals. In a subsequent study, the same authors also demonstrated that altrenogest prevented cloprostenol-induced abortion at 80-150 days of gestation. McKinnon and co-workers had similar results with altrenogest, but failed to prevent abortion with other progestins after prostaglandin-induced luteolysis in early gestation,[69] while Vanderwall and co-workers were able to prevent abortion in cloprostenol-treated mares with both altrenogest and a compounded injectable progesterone formulation.[70] Likewise, in an experimental model of placentitis, treatment with a combination of TMS, PTX and ALT improved foal survival, whereas treatment with TMS and PTX was unable to do so.[20,35] Thus, although the mechanism of action is not well-characterized, the use of a progestin to treat preterm labor and placentitis is strongly supported in both women and mares.

Conclusion

In conclusion, despite substantial research investments into this condition over the past three decades, placentitis remains a significant problem to the equine breeding industry. Further, while recent work from numerous laboratories has made headway in developing a more complete understanding of the pathophysiology of disease and treatment effect, this has not translated to substantial clinical improvements in cases of naturally-occurring placentitis. One key challenge that remains is to develop early, sensitive diagnostic tools that are cost-effective and can be used to screen mares during mid and late gestation. Recent work by da Silva and other groups suggests that SAA may serve this purpose.[23,32] A second challenge that remains is to identify effective anti-inflammatory therapy. A recent publication by Gravett and coworkers suggests that in human preterm disease, inflammation may result in more fetal damage than infection.[19] Lastly, achieving bacterial clearance from the fetus, fetal fluids and uterus within a short period of time after diagnosis will likely be a key component of treatment success and the prevention of antimicrobial resistance and secondary infections.

References

1. Giles RC, Donahue JM, Hong CB, et al: Causes of abortion, stillbirth, and perinatal death in horses - 3,527 cases (1986-1991). J Am Vet Med Assoc 1993;203:1170-1175.
2. Hong CB, Donahue JM, Giles RC, et al: Equine abortion and stillbirth in central kentucky during 1988 and 1989 foaling seasons. J Vet Diagn Invest 1993;5:560-566.
3. Laugier C, Foucher N, Sevin C, et al: A 24-year retrospective study of equine abortion in normandy (France). J Equine Vet Sci 2011;31:116-123.
4. Quinn FD: An emerging veterinary and potentially zoonotic streptococcus. Vet J 2011;188:132-133.

5. Edwards A, Roulson M, Ironside M. A milk-borne outbreak of serious infection due to *Streptococcus zooepidemicus* (Lancefield group-C). Epidemiol Infect 1988;101:43-51.
6. Chalker V, Brooks H, Brownlie J: The association of *Streptococcus equi* subsp *zooepidemicus* with canine infectious respiratory disease. Vet Microbiol 2003;29:95:149-156.
7. Jovanovic M, Stevanovic G, Tosic T, et al: *Streptococcus equi* subsp *zooepidemicus* meningitis. J Med Microbiol 2008;57:373-375.
8. Ousey JC, Fowden AL: Prostaglandins and the regulation of parturition in mares. Equine Vet J 2012;44:140-148.
9. Fowden AL, Forhead AJ, Ousey JC: Endocrine adaptations in the foal over the perinatal period. Equine Vet J 2012;44:130-139.
10. Fowden AL, Forhead AJ, Ousey JC: The endocrinology of equine parturition. Exp Clinical Endocrinol Diabetes 2008;116:393-403.
11. Challis J, Patel F, Pomini F: Prostaglandin dehydrogenase and the initiation of labor. J Perinat Med 1999;27:26-34.
12. Olson D, Skinner K, Challis J: Prostaglandin synthesis regulation during human parturition [abstract]. Fertil Steril 1982;38:134.
13. LeBlanc MM, Giguere S, Lester GD, et al: Relationship between infection, inflammation and premature parturition in mares with experimentally induced placentitis. Equine Vet J 2012;44:8-14.
14. Lyle SK, Paccamonti DL, Hubert JD, et al. Laparoscopic placement of an indwelling allantoic catheter in the mare: biochemical, cytologic, histologic, and microbiologic findings. Anim Reprod Sci 2006;94:428-431.
15. Lyle SK, Hague M, Lopez MJ, et al. *In vitro* production of cortisol by equine fetal adrenal cells in repsonse to ACTH and IL-1b. Anim Reprod Sci 2010;121:322.
16. Chavatte P, Rossdale P, Tait A: Modulation of 3-beta-hydroxysteroid dehydrogenase (3-beta-HSD) activity in the equine placenta by pregnenolone and progesterone metabolites. Equine Vet J 1995;27:342-347.
17. Patel F, Challis J: Cortisol/progesterone antagonism in regulation of 15-hydroxysteroid dehydrogenase activity and mRNA levels in human chorion and placental trophoblast cells at term. J Clin Endocrinol Metab 2002;87:700-708.
18. Combs CA, Gravett M, Garite T, et al: Intramniotic inflammation may be more important than the presence of microbes as a determinant of perinatal outcome in preterm labor. Obstet Gynecol 2013;208:S44-S44.
19. McAdams RM, Vanderhoeven J, Beyer RP, et al: Choriodecidual infection downregulates angiogenesis and morphogenesis pathways in fetal lungs from *Macaca nemestrina*. PLoS One 2012;7(10):e46863.
20. Bailey CS, Macpherson ML, Pozor MA, et al. Treatment efficacy of trimethoprim sulfamethoxazole, pentoxifylline and altrenogest in experimentally induced equine placentitis. Theriogenology 2010;74:402-412.
21. Zent WW, Williams NM, Donahue JM: Placentitis in Central Kentucky broodmares. Pferdeheilkunde 1999;15:630-632.
22. Renaudin CD, Troedsson MH, Gillis CL, et al: Ultrasonographic evaluation of the equine placenta by transrectal and transabdominal approach in the normal pregnant mare. Theriogenology 1997 Jan 15;47:559-573.
23. Coutinho da Silva MA, Canisso IF, Macpherson ML, et al: Serum amyloid A concentration in healthy periparturient mares and mares with ascending placentitis. Equine Vet J 2012 Dec 20. doi:10.1111/evj.12034[Epub ahead of print].
24. Bailey CS, Heitzman JM, Buchanan CN, et al: B-mode and Doppler ultrasonography in pony mares with experimentally induced ascending placentitis. Equine Vet J 2012;44:88-94.
25. Ousey JC: Hormone profiles and treatments in the late pregnant mare. Vet Clin North Am Equine Pract 2006;22:727-247.
26. Santschi E, LeBlanc ML Fetal and placental conditions that cause high-risk pregnancy in mares. Compend Contin Educ Pract Vet 1995;17:710-721.
27. Stawicki R, Ruebel H, Hansen P, et al: Endocrinological findings in an experimental model of ascending placentitis in the mare. Theriogenology 2002;58:849-852.
28. Ousey J, Houghton E, Grainger L, et al: Progestagen profiles during the last trimester of gestation in Thoroughbred mares with normal or compromised pregnancies. Theriogenology 2005;63:1844-1856.
29. Morris S, Kelleman AA, Stawicki RJ, et al: Transrectal ultrasonography and plasma progestin profiles identifies feto-placental compromise in mares with experimentally induced placentitis. Theriogenology 2007;67:681-691.
30. Gravett M, Nagalla S, Rosenfeld R, et al: Diagnosis of intraamniotic infection by proteomic identification of novel biomarkers. J Soc Gynecol Investig 2004;11:308A-309A.
31. Ticconi C, Belmonte A, Piccione E,et al: Feto-placental communication system with the myometrium in pregnancy and parturition: the role of hormones, neurohormones, inflammatory mediators, and locally active factors. J Matern Fetal Neonatal Med 2006;19:125-133.
32. Canisso IF, Troedsson MHT, Cray C, et al. Serum amyloid A and haptoglobulin concentrations in pregnant mares with experimentally induced ascending placentitis. Proc Annu Conv Am Assoc Equine Pract 2012. p. 524-525.
33. Suagee JK, Corl BA, Crisman MV, et al: Relationships between body condition score and plasma inflammatory cytokines, insulin, and lipids in a mixed population of light-breed horses. J Vet Intern Med 2013;27:157-163.
34. Macpherson ML, Bailey CS: Treating the mare with placentitis: a clinical approach. J Equine Vet Sci 2008;28:703-708.
35. Graczyk J, Macpherson ML, Pozor MA, et al: Treatment efficacy of trimethoprim sulfamethoxazole and pentoxifylline in equine placentitis. Anim Reprod Sci 2006;94:434-435.
36. Rebello SA, Macpherson ML, Murchie TA, et al: Placental transfer of trimethoprim sulfamethoxazole and pentoxifylline in pony mares. Anim Reprod Sci 2006;94:432-433.

37. Murchie TA, Macpherson ML, LeBlanc MM, et al: Continuous monitoring of penicillin G and gentamicin in allantoic fluid of pregnant pony mares by in vivo microdialysis. Equine Vet J 2006;38:520-525.
38. Macpherson ML, Giguere S, Hatzel JN, et al. Disposition of desfuroylceftiofur acetamide in serum, placental tissue, fetal fluids, and fetal tissues after administration of ceftiofur crystalline free acid (CCFA) to pony mares with placentitis. J Vet Pharmacol Ther 2013;36:59-67.
39. Graczyk J, Macpherson ML, Pozor MA, et al: Treatment efficacy of trimethoprim sulfamethoxazole and pentoxifylline in equine placentitis. Anim Reprod Sci 2006;94:434-435.
40. Ensink JM, Klein WR, Barneveld A, et al: Distribution of penicillins into subcutaneous tissue chambers in ponies. J Vet Pharmacol Ther 1996;19:439-444.
41. Ensink JM, Smit JAH, van Duijkeren E: Clinical efficacy of trimethoprim/sulfadiazine and procaine penicillin G in a *Streptococcus equi* subsp *zooepidemicus* infection model in ponies. J Vet Pharmacol Ther 2003;26:247-252.
42. Macpherson M: Treatment strategies for mares with placentitis. Theriogenology 2005;64:528-534.
43. Macpherson ML: Diagnosis and treatment of equine placentitis. Vet Clin North Am Equine Pract 2006;22:763-776.
44. Diaw M, Bailey CS, Schlafer DH, et al: Evaluation of endometrial culture and biopsy immediately post partum from normal mares and those with induced placentitis. Anim Reprod Sci 2010;121:369-370.
45. Daels PF, Stabenfeldt GH, Hughes JP, et al: Effects of flunixin meglumine on endotoxin-induced prostaglandin-f2-alpha secretion during early-pregnancy in mares. Am J Vet Res 1991;52:276-281.
46. Nandi J, Saud B, Zinkievich JM, et al: TNF-alpha modulates iNOS expression in an experimental rat model of indomethacin-induced jejunoileitis. Mol Cell Biochem 2010;336:17-24.
47. Barton M, Ferguson D, Davis P, et al: The effects of pentoxifylline infusion on plasma 6-keto-prostaglandin F-1 alpha and ex vivo endotoxin-induced tumour necrosis factor activity in horses. J Vet Pharmacol Ther 1997;20:487-492.
48. Baskett A, Barton MH, Norton N, et al: Effect of pentoxifylline, flunixin meglumine, and their combination on a model of endotoxemia in horses. Am J Vet Res 1997;58:1291-1299.
49. Lauterbach R, Grabowska A, Marcinkiewicz J: Effect of pentoxifylline on nitric-oxide released by murine macrophages. Biol Neonate 1995;67:72-76.
50. Lauterbach R, Rytlewski K, Pawlik D, et al: Effect of pentoxifylline, administered in preterm labour, on the foetal-placental circulation and neonatal outcome: a randomized, prospective pilot study. Basic Clin Pharmacol Toxicol 2012;110:342-346.
51. Ousey JC, Koelling M, Willis D, et al: Effects of pentoxifylline on uterine blood flow, and placental and fetal development in young and aged mares with endometrosis. Anim Reprod Sci 2010;121S:343-344.
52. Ousey JC, Koelling M, Newton R, et al: Uterine haemodynamics in young and aged pregnant mares measured using Doppler ultrasonography. Equine Vet J 2012;44:15-21.
53. Bailey CS, Sper RB, Schewmaker JL, et al: Uterine artery blood flow remains unchanged in pregnant mares in response to short-term administration of pentoxifylline. Theriogenology 2012;77:430-436.
54. Sadowsky D, Novy M, Witkin S, et al: Dexamethasone or interleukin-10 blocks interleukin-1 beta-induced uterine contractions in pregnant rhesus monkeys. Obstet Gynecol 2003;188:252-263.
55. Gravett MG, Adams KM, Sadowsky DW, et al: Immunomodulators plus antibiotics delay preterm delivery after experimental intraamniotic infection in a nonhuman primate model. Obstet Gynecol 2007;197:518.e1-8.
56. Ousey JC, Kolling M, Kindahl H, et al: Maternal dexamethasone treatment in late gestation induces precocious fetal maturation and delivery in healthy Thoroughbred mares. Equine Vet J 2011;43:424-429.
57. Ousey JC, Kolling M, Allen WR: The effects of maternal dexamethasone treatment on gestation length and foal maturation in Thoroughbred mares. Anim Reprod Sci 2006;94:436-438.
58. Christiansen DL, Moulton K, Hopper RM, et al. Evidenced-based medicine approach to develop efficacious therapies for late-gestation mares presenting with uterine infections using an experimentally-induced placentitis model. Anim Reprod Sci 2010;121(Suppl):345-346.
59. James MFM: Magnesium in obstetrics. Best Pract Res Clin Obstet Gynaecol 2010;24:327-337.
60. Rath W. Tocolysis: open questions unsolved problems future perspectives. Geburtshilfe Frauenheilkd 2009;69:677-681.
61. Palmer E, Chavatte-Palmer P, Duchamp G, et al: Lack of effect of clenbuterol for delaying parturition in late pregnant mares. Theriogenology 2002;58:797-799.
62. Garfield RE, Rabideau S, Challis JRG, et al: Hormonal-control of gap junction formation in sheep myometrium during parturition. Biol Reprod 1979;21:999-1007.
63. Bishop CV, Stormshak F: Non-genomic actions of progesterone and estrogens in regulating reproductive events in domestic animals. Vet J 2008;176:270-280.
64. Meis P, Soc Maternal-Fetal Med: 17 hydroxyprogesterone for the prevention of preterm delivery. Obstet Gynecol 2005;105:1128-1135.
65. Palacio M, Cobo T, Antolin E, et al: Vaginal progesterone as maintenance treatment after an episode of preterm labor (PROMISE study): a randomized, double blinded, placebo-controlled trial. Obstet Gynecol 2013;208:S10-S11.
66. Maher MA, Abdelaziz A, Ellaithy M, et al: Prevention of preterm birth: a randomized trial of vaginal compared with intramuscular progesterone. Acta Obstet Gynecol Scand 2013;92:215-222.
67. Daels PF, Besognet B, Hansen B, et al: Effect of progesterone on prostaglandin F-2 alpha secretion and outcome of pregnancy during cloprostenol-induced abortion in mares. Am J Vet Res 1996;57:1331-1337.

68. Daels PF, Stabenfeldt GH, Hughes JP, et al: Evaluation of progesterone deficiency as a cause of fetal death in mares with experimentally induced endotoxemia. Am J Vet Res 1991;52:282-288.
69. McKinnon AO, Figueroa STD, Nobelius AM, et al: Failure of hydroxyprogesterone caproate to maintain pregnancy in ovariectomized mares. Equine Vet J 1993;25:158-160.
70. Vanderwall DK, Marquardt JL, Woods GL: Use of a compounded long-acting progesterone formulation for equine pregnancy maintenance. J Equine Vet Sci 2007;27:62-66.

Metabolic diseases – do we really know if they affect reproduction?

Peter R. Morresey
Rood and Riddle Equine Hospital, Lexington, KY

Abstract

Conditions affecting metabolism (including endocrine dysfunction) intuitively have wide-ranging effects on potentially all body functions. In many species metabolic and endocrinological changes have been proven to have significant effects on female reproductive processes, with ovarian activity, establishment of pregnancy, pregnancy maintenance to term and the overall health of the fetus impaired. Effects on the reproductive competence of the male are also known to occur. With respect to the conceptus itself, life-long alterations in metabolic processes are found in the offspring of affected females when compared to non-affected cohorts. The presence of insulin resistance with hyperinsulinemia and the subsequent generation of a chronic proinflammatory state appears to be the genesis of these problems. In horses details regarding the effects of metabolic and endocrine dysfunction on reproductive competence remains largely unknown however potential for alteration can be inferred due to similarities in reproductive physiology between species. Clinical observations of individuals support the likelihood of metabolic and endocrine dysfunction detrimentally affecting equine reproductive efficiency.

Keywords: Metabolic, endocrine, pituitary, hyperinsulinemia, proinflammatory. cyclicity, pregnancy

Introduction

Metabolic syndrome describes a set of clinical findings in human patients including insulin resistance, hyperinsulinemia, dyslipidemia, hypertension and atherosclerosis, however obesity while common is not a consistent finding. A chronic pro-inflammatory condition is thought to exist in metabolic syndrome. Metabolic syndrome was introduced as a diagnostic category to identify human individuals at risk of developing type 2 diabetes and atherothrombotic cardiovascular disease.[1] This term has come to be applied to equines suffering from obesity, dyslipidemia, insulin resistance and hyperinsulinemia.

Pituitary pars intermedia dysfunction (PPID, or more commonly Cushing's syndrome) is a common condition of aged horses and ponies in which inhibition of the intermediate lobe of the pituitary is lost due to degeneration of the hypothalamic dopaminergic neurons. During the early stages of this syndrome cellular hyperplasia of the intermediate lobe results in increasing levels of pro-opiomelanocortin (POMC), a precursor molecule cleaved into many peptides including alpha-melanocyte stimulating hormone (α-MSH), adrenocorticotropic hormone (ACTH), beta-endorphin and corticotropin-like intermediate lobe peptide (CLIP). As the syndrome progresses the pars intermedia enlarges until a singular intermediate lobe adenoma develops that compresses the adjacent pituitary and hypothalamic structures. Furthermore, a relationship between the onset of metabolic syndrome and PPID is anecdotally reported. Many PPID horses are insulin resistant, this being theorized to be a result of obesity, chronic hyperinsulinemia, or insulin resistance.

What is going on in metabolic syndrome and PPID?

Insulin resistance was first proposed as a cause of glucose intolerance, elevated insulin levels, dyslipidemia, and hypertension in humans over 20 years ago.[2] Metabolic syndrome has developed from this concept, being defined as a combination of cardiovascular risk factors including such diverse components as visceral adipose accumulation, insulin resistance, hyperinsulinemia, hypertension, chronic inflammation, microalbuminemia, and a prothrombotic disorder leading to endothelial cell dysfunction and atherosclerosis.[3]

Adipose tissue is not simply a store of excess energy, but is rather an organ of diverse functions which plays a pivotal role in development of metabolic syndrome.[4] Adipokines, biologically active secretions of adipose tissue, may play a role in the pathogenesis of insulin resistance as some have been

shown to have effects on insulin sensitivity and signaling.[5] It is the resulting hyperinsulinemic proinflammatory state that is thought to drive the laminitis seen in affected horses.

Considerable debate exists regarding the etiology and pathogenesis of metabolic syndrome as no single unifying mechanism has as yet been elucidated.[6] A complex interaction between genetics, hormonal status and nutrition is most likely. The concept of metabolic syndrome in the horse while relatively new is widely researched and reviewed.[7]

In PPID, following loss of hypothalamic dopaminergic inhibition acting on the pituitary, increased synthesis of POMC peptides and proliferation of melanocytes occurs. Increased ACTH secretion can lead to hypercortisolemia which has profound effects on metabolic and immune function. It is thought the altered secretion of prolactin and the gonadotrophic hormones may potentially be responsible for effects on reproductive function. Hypertrichosis, chronic infections, loss of muscle mass, abnormal fat deposition, slow wound healing, polyuria and polydipsia, and lethargy are the common clinical signs. Many PPID horses are also insulin resistant.

Additionally, horses with metabolic syndrome or PPID may be clinically indistinguishable in the early stages of either condition as both may display abnormal adiposity and hyperinsulinemia with hyperglycemia.

Are there proven reproductive effects of metabolic syndrome or PPID in other species?

Ovarian function

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders in women of reproductive age, and is characterized by hyperandrogenic chronic anovulation.[8] The PCOS is considered a pre-diabetic state with affected women displaying features of metabolic syndrome including insulin resistance, obesity, and dyslipidemia, suggesting a heightened risk for cardiovascular disease.[9] Insulin has a major role in the regulation of ovarian steroidogenesis, follicular development and granulosa cell proliferation.[10] Insulin resistance has been recognized as the major factor related to PCOS, with compensatory hyperinsulinemia stimulating ovarian androgen production and the peripheral aromatization of androgens to estrogens, altering gonadotropin secretion and therefore follicular development.[11]

Pregnancy

The PCOS has been associated with the development of gestational diabetes with affected women entering pregnancy with increased insulin resistance compared to normal women.[12] Subjects that have PCOS prior to the onset of pregnancy have increased rates of first trimester spontaneous abortion compared to unaffected women.[13]

Human pregnancy raises requirements for insulin secretion simultaneously with increasing insulin resistance, raising synthetic demands on pancreatic beta cells and promoting development of gestational diabetes.[14,15] With advancing pregnancy the response of insulin to glucose increases, however sensitivity to insulin decreases.[16,17] Insulin sensitivity in healthy pregnant women was found to be reduced by two-thirds when compared to that of non-pregnant women.[18] The resulting physiological insulin resistance promotes transfer of glucose from the mother to the fetus.[18] By this means, the body is prepared for the impending demands for growth of the placenta and fetus.

The human male

Significant hormonal differences occur between subfertile obese and fertile males of normal body weight.[19] Obesity has been shown to effect sperm production with sperm concentration decreased in highly obese men, and a continuous decrease associated with advancing age.[20] Total testosterone is decreased with obesity, however estradiol is not affected leading to a decrease in the testosterone:estradiol ratio which influences the hypothalamic-pituitary axis.[21] Resulting suppression of testicular function can therefore feed back in a self-reinforcing cycle. Furthermore, the relative decrease in muscle mass and

increase in body fat resulting from decreased testosterone promotes obesity, insulin resistance, and further functional pituitary suppression.[22]

Metabolic syndrome and the mare

Ovarian function

Metabolic syndrome affects the insulin-like growth factor system, this being crucial to follicle selection and dominance in the mare, leading to disturbances of ovarian function.[23,24] Obese mares with reduced insulin sensitivity have been shown to have prolonged interovulatory and luteal phases.[25]

What is happening in the normal late pregnant mare?

Marked changes in carbohydrate metabolism and pancreatic beta cell function occur during pregnancy in the mare, in common with other researched species. Pregnant mares consuming high starch feeds in the third trimester have increased insulin and glycemic responses to feeding than non-pregnant mares or matched pregnant mares consuming a fat and fiber based diet.[26] Hyperinsulinemia, increased pancreatic □ cell sensitivity to glucose, and increased resistance to the action of insulin occur.[16]

Glucose uptake by the fetoplacental unit is dependent solely on the concentration gradient between the maternal and fetal circulations across the placenta.[27] No increase in glucose uptake by the fetus occurs during pregnancy; the increased requirements for growth and development during gestation are met solely by redirection from maternal tissues. Following periods of fasting, the sensitivity to glucose of the pancreatic beta cells is reduced, this allowing preferential transfer of glucose to the fetus by limiting maternal uptake.[16]

Up to approximately 270 days of gestation, enhanced pancreatic beta cell sensitivity to glucose results in hyperinsulinemia.[16] This allows both fetal and maternal requirements to be met without inducing hypoglycemia. Following this period, the fetus gains approximately 45% of its final birth weight and consequently has a high absolute glucose demand.[28] Uterine glucose uptake removes 75% of that lost from the maternal circulating pool.[29] Maternal glucose usage is therefore reduced to a minimum to allow this transfer to the developing fetus. Insulin concentrations and pancreatic □ cell sensitivity to glucose are also reduced compared to earlier in gestation.[16] It should therefore now be realized that insulin resistance is a normal occurrence in the pregnant mare, one that enables redirection of maternal nutrients to meet the high demands the developing fetus.

Effects of metabolic syndrome on the maintenance of equine pregnancy

The effects on pregnancy of metabolic syndrome in the mare are not proven, however in humans gestational diabetes, abortion and fetal compromise have been reported.[1213] Interestingly, a reduction is seen in first trimester spontaneous abortion in women with metformin usage,[30] and no evidence of teratogenic effects on the fetus with this approach has been reported.[31,32] An improvement in early pregnancy rates has been reported with the use of metformin in mares showing clinical signs compatible with metabolic syndrome (Dr. Kristina Lu, personal communication).

Metabolic syndrome and the stallion

As in the mare, reproductive effects of metabolic syndrome have not been clearly defined, although there is a wealth of comparative information available. It has been shown that there is an interaction between metabolic syndrome and testicular function that is bidirectional in nature. Obesity can affect spermatogenesis and alterations in the hypothalamic-pituitary testicular axis occur. Resulting endocrine function changes in the testes have been shown to impact systemic metabolic parameters.

PPID and reproductive disorders

Mares with PPID may suffer from persistent uterine infections that are challenging to clear. However cyclical activity may continue and mares without uterine infection can be bred successfully. In others, cyclic irregularities (anestrus, delayed ovulation) may be present. If these mares are bred,

anecdotal reports indicate a lower likelihood of carriage to term and delivery of a healthy foal. In non-pregnant mares, serous mammary secretions may occur regardless of previous lactation status, with this theorized to be a manifestation of excessive prolactin secretion.

The use of the dopamine agonist pergolide may allow restoration of appropriate cyclicity and a pregnancy may be established. If carried to term, agalactia following delivery (due to prolactin antagonism) is possible. As a result, it is a common recommendation that pergolide therapy be discontinued between two and four weeks prior to the expected foaling date. Anecdotally, the author and others have maintained pregnant mares on pergolide throughout gestation without detriment to foal delivery or adequate milk production. Information on the reproductive effects of PPID in stallions is not available.

Summary

The ability to quantify the reproductive effects of metabolic syndrome and PPID is limited due to the difficulties in definitively diagnosing either condition at this time. However, consideration of comparative reproductive physiology strongly supports anecdotal observations in the horse regarding disturbances in cyclicity, pregnancy establishment and maintenance, and spermatogenesis as a result of metabolic or hypothalamic-pituitary disturbances.

References

1. Reaven GM: The metabolic syndrome: requiescat in pace. Clin Chem 2005;51:931-938.
2. Reaven GM: Banting lecture 1988. Role of insulin resistance in human disease. Diabetes 1988;37:1595-607.
3. Bruce KD, Hanson MA: The developmental origins, mechanisms, and implications of metabolic syndrome. J Nutr 2010;140:648-652.
4. Burns TA, Geor RJ, Mudge MC, et al: Proinflammatory cytokine and chemokine gene expression profiles in subcutaneous and visceral adipose tissue depots of insulin-resistant and insulin-sensitive light breed horses. J Vet Intern Med 2010;24:932-939.
5. Kershaw EE, Flier JS: Adipose tissue as an endocrine organ. J Clin Endocrinol Metab 2004;89:2548-2556.
6. Ferrannini E. Metabolic syndrome: a solution in search of a problem. J Clin Endocrinol Metab 2007;92:396-398.
7. Frank N, Geor RJ, Bailey SR, et al: Equine metabolic syndrome. J Vet Intern Med 2010;24:467-475.
8. Azziz R, Woods KS, Reyna R, et al: The prevalence and features of the polycystic ovary syndrome in an unselected population. J Clin Endocrinol Metab 2004;89:2745-2749.
9. Sharpless JL. Polycystic ovary syndrome and the metabolic syndrome. Clin Diabetes 2003;21:154-161.
10. Willis D, Mason H, Gilling-Smith C, et al: Modulation by insulin of follicle-stimulating hormone and luteinizing hormone actions in human granulosa cells of normal and polycystic ovaries. J Clin Endocrinol Metab 1996;81:302-309.
11. Diamanti-Kandarakis E, Bergiele A: The influence of obesity on hyperandrogenism and infertility in the female. Obes Rev 2001;2:231-238.
12. Lanzone A, Fulghesu AM, Cucinelli F, et al: Preconceptional and gestational evaluation of insulin secretion in patients with polycystic ovary syndrome. Hum Reprod 1996;11:2382-2386.
13. Glueck CJ, Wang P, Goldenberg N, et al: Pregnancy outcomes among women with polycystic ovary syndrome treated with metformin. Hum Reprod 2002;17:2858-2864.
14. Glueck CJ, Goldenberg N, Streicher P, et al: The contentious nature of gestational diabetes: diet, insulin, glyburide and metformin. Expert Opin Pharmacother 2002;3:1557-1568.
15. Glueck CJ, Streicher P, Wang P: Treatment of polycystic ovary syndrome with insulin-lowering agents. Expert Opin Pharmacother 2002;3:1177-1189.
16. Fowden AL, Comline RS, Silver M: Insulin secretion and carbohydrate metabolism during pregnancy in the mare. Equine Vet J 1984;16:239-246.
17. Zavalza-Gomez AB, Anaya-Prado R, Rincon-Sanchez AR, et al: Adipokines and insulin resistance during pregnancy. Diabetes Res Clin Pract 2008;80:8-15.
18. Buchanan TA, Metzger BE, Freinkel N, et al: Insulin sensitivity and B-cell responsiveness to glucose during late pregnancy in lean and moderately obese women with normal glucose tolerance or mild gestational diabetes. Am J Obstet Gynecol 1990;162:1008-1014.
19. Jarow JP, Kirkland J, Koritnik DR, et al: Effect of obesity and fertility status on sex steroid levels in men. Urology 1993;42:171-174.
20. Koloszar S, Fejes I, Zavaczki Z, et al: Effect of body weight on sperm concentration in normozoospermic males. Arch Androl 2005;51:299-304.
21. Fejes I, Koloszar S, Zavaczki Z, et al: Effect of body weight on testosterone/estradiol ratio in oligozoospermic patients. Arch Androl 2006;52:97-102.

22. Cohen PG. Aromatase, adiposity, aging and disease. The hypogonadal-metabolic-atherogenic-disease and aging connection. Med Hypotheses 2001;56:702-708.
23. Beg MA, Ginther OJ: Follicle selection in cattle and horses: role of intrafollicular factors. Reproduction 2006;132:365-377.
24. Ginther OJ, Gastal EL, Gastal MO, et al: Critical role of insulin-like growth factor system in follicle selection and dominance in mares. Biol Reprod 2004;70:1374-1379.
25. Vick MM, Sessions DR, Murphy BA, et al: Obesity is associated with altered metabolic and reproductive activity in the mare: effects of metformin on insulin sensitivity and reproductive cyclicity. Reprod Fertil Dev 2006;18:609-617.
26. George LA, Staniar WB, Cubitt TA, et al: Evaluation of the effects of pregnancy on insulin sensitivity, insulin secretion, and glucose dynamics in Thoroughbred mares. Am J Vet Res 2011;72:666-674.
27. Simmons MA, Battaglia FC and Meschia G: Placental transfer of glucose. J Dev Physiol 1979;1:227-243.
28. Meyer H, Ahlswede L: The intra-uterine growth and body composition of foals and the nutrient requirements of pregnant mares. Anim Res Dev 1978;8:86-112.
29. Evans JW: Effect of fasting, gestation, lactation and exercise on glucose turnover in horses. J Anim Sci 1971;33:1001-1004.
30. Glueck CJ, Phillips H, Cameron D, et al: Continuing metformin throughout pregnancy in women with polycystic ovary syndrome appears to safely reduce first-trimester spontaneous abortion: a pilot study. Fertil Steril 2001;75:46-52.
31. Coetzee EJ, Jackson WP: Oral hypoglycaemics in the first trimester and fetal outcome. S Afr Med J 1984;65:635-637.
32. Coetzee EJ, Jackson WP: Pregnancy in established non-insulin-dependent diabetics. A five-and-a-half year study at Groote Schuur Hospital. S Afr Med J 1980;58:795-802.

The use of endometrial biopsies in formulating a treatment plan

Steven P. Brinsko,[a] Patricia L. Sertich[b]
[a]Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Texas A&M University, College Station, TX; [b] Department of Clinical Studies-New Bolton Center, School of Veterinary Medicine, University of Pennsylvania, Kennett Square, PA

Introduction

Histologic descriptions of the mare's endometrium were reported at least as early as the 1920's.[1,2] However, it was not until the publications and classifications proposed by Robert Kenney[3,4] and Sidney Ricketts[5,6] in the mid- to late 1970's that use of endometrial biopsy for diagnostic and prognostic purposes in the mare was widely appreciated. Following this and the development of suitable instruments, endometrial biopsy sample submission by practitioners for histological evaluation has been primarily used to help diagnose the contribution of the endometrium to infertility or embryonic/fetal loss, as an integral part of the breeding soundness examination, and as a screening tool in pre-purchase examinations as well as for assessing the suitability of mares as embryo donors and recipients. The histological evaluation of the endometrium is also a way to monitor patient response to therapy when uterine infections or other endometrial abnormalities are diagnosed and treated. An improvement in the biopsy score following treatment (i.e., disappearance or decrease in the severity of pathological changes detected in a previous endometrial biopsy) has been shown to be more closely related to the subsequent fertility of mares than simply basing a prognosis for fertility on the histological assessment of a single pre-treatment biopsy sample.[7] One of the most valuable, yet underutilized benefits of endometrial biopsy evaluation is in formulating a treatment plan prior to initiating therapy.

Key Words: Endometrial biopsy, mare, treatment plan

Biopsy sample procurement

Detailed descriptions of how to obtain an endometrial biopsy are reported elsewhere.[8,9] Briefly, the procedure for acquiring an endometrial biopsy is the same as that for procuring an endometrial swabbing for culture, except that the closed biopsy instrument is passed through the cervix to the base of one of the uterine horns so that a representative specimen of endometrium is obtained. Endometrial specimens obtained too close to the cervix have reduced glandular density and shallow gland penetration into the lamina propria, which can impede accurate assessment of normalcy or pathology. After inserting the biopsy instrument into the uterus, the gloved hand is inserted into the rectum while the other hand holds the biopsy instrument handle. Using the hand in the rectum, the biopsy punch can be guided to the base of one of the uterine horns or another location of interest as determined by palpation or ultrasonographic findings. The biopsy jaws are opened and the endometrium is pressed into the jaws which are then closed, to procure the sample of the endometrium. If more precision is needed, a video endoscope with a biopsy channel can be used to identify and obtain the biopsy from a specific area of the endometrium. Once the endometrial sample is obtained, the biopsy instrument is withdrawn from the mare's reproductive tract, and the endometrial specimen is placed in a suitable fixative, such as Bouin's solution, Davidson's solution, or 10% buffered formalin and transported to a reference laboratory for processing and histological interpretation. After sectioning and embedding the biopsy tissue into paraffin, a variety of stains can be used to detect histologic changes.

Using endometrial interpretation in the therapeutic plan

Many abnormalities which have the potential to adversely affect a mare's fertility can only be detected by histological evaluation of an endometrial biopsy. Examples of these include periglandular fibrosis, cystic glandular distension, lymphatic distension, and chronic inflammatory changes within the endometrium.

Mares with significant lymphatic lacunae are more likely to have endometrial cysts that may or may not adversely affect fertility, depending on their number, size, distribution and location. More

importantly, these lesions may be a symptom of a deeper underlying problem such as poor uterine contractility and their identification can alert the practitioner to the need for post-breeding administration of ecbolics or uterine lavage to help prevent intrauterine fluid accumulations and persistent mating induced endometritis. An appreciation of the extent and severity of endometrial lesions can also help practitioners and their clients make better informed decisions on whether or not to pursue genital tract surgeries. For example, if an older mare is in need of a urethral extension to correct urine pooling and is found to have a Category III endometrium due to extensive periglandular fibrosis, it is unlikely that surgery to correct urine pooling would have a significant effect on improving the mare's chances of becoming pregnant and carrying a foal to term. Conversely, a mare with only minimal to moderate endometrial pathology or even severe pathology that is amendable to treatment may be deemed a good surgical candidate. Likewise, decisions, based on endometrial quality, can be made on whether or not to perform salvage procedures to maintain the animal as a broodmare.

Many practitioners submit their endometrial biopsies to laboratories where interpretations are performed by a general pathologist. Although the pathological changes in an endometrial biopsy can be described by a general pathologist, determination of the clinical significance of these findings requires interpretation by a clinician with experience in equine reproduction. As Schlafer points out in a recent review,[10] the pioneers in endometrial interpretation had advanced training in pathology and clinical equine reproduction and thus were uniquely positioned to be able to evaluate microscopic changes in the context of the clinical status and subsequent reproductive performance of the individual mare. While a pathologist can provide a description of the histologic findings in the biopsy and even a prognostic categorization (typically based on the modified Kenney-Doig system; I, IIa, IIb, and III),[11] the theriogenologist also includes an epicrisis to provide the practitioner with a practical prognosis and general guidelines for therapy. The theriogenologist can also comment on the potential for category reclassification should therapy be effective. Providing pertinent clinical information when the biopsy sample is submitted greatly enhances the ability of the theriogenologist to provide a meaningful epicrisis.

Too often the decision on whether to purchase, breed or treat a mare is based primarily on the endometrial biopsy category alone. It is important to realize that additional valuable information is present in a report and that all mares within a category are not equal. The histologic changes that cause the mare's endometrium to be placed in a particular category are the aspects upon which the practitioner should focus. Mares with a category III biopsy are often considered unsalvageable or not worthy of treatment because they are considered to have a 10% or less chance of becoming pregnant and carrying a foal to term. If the category III is based primarily upon the degree of endometrial fibrosis, this assumption may be justified. However, it is not uncommon for a mare's biopsy to be a category III based solely upon widespread, diffuse, severe inflammation. If the latter is the case and appropriate therapeutic measures are undertaken which resolves the inflammation, a subsequent biopsy may be upgraded in category with an attendant increase in fertility. For both the practitioner and the mare owner, it is important for the epicrisis to include this vital piece of information.

When inflammation is diagnosed on an endometrial biopsy, the nature of the inflammation can be used to formulate a therapeutic plan. Characteristics such as the type of inflammation (acute, subacute, chronic), its distribution (widespread, scattered, diffuse, focal, superficial, deep, etc.), severity (mild, moderate, severe) and the various combinations thereof indicate the need for different therapeutic strategies. Widespread, diffuse, severe inflammation throughout the lamina propria warrants a longer duration of therapy than would superficial, mild to moderate inflammation. Observance of inflammatory cells and exudate in the luminal contents or adherent to the luminal epithelium would strongly support the need for uterine lavage to be used in conjunction with appropriate antimicrobial therapy based on culture and sensitivity. Lavage of the uterus prior to the infusion of antibiotics may be of benefit in removing bacteria and debris that may interfere with antimicrobial activity. Uterine lavage may also be of benefit by increasing uterine tone and decreasing cystic gland distension or lymphatic stasis when these lesions are observed. The presence of eosinophils and/or plasma cells in conjunction with epithelial pleomorphism, suggests a need to address the mare's perineal conformation and/or cervical integrity,

including the potential for urine pooling. Culture of endometrial biopsy samples has been shown to be more accurate for detecting the presence of bacteria than those obtained from uterine swabs.[12]

Yeast and fungal infections may result in chronic deep endometritis that responds poorly to treatment. However, many yeast and fungal infections that are superficial will readily respond to treatment. Surprisingly, yeast and fungal infections do not always result in a significant inflammatory response observable on endometrial biopsy samples. However, special stains such as Gomori's methenamine silver (GMS) or periodic acid-Schiff (PAS) can be used to identify the organisms in the tissue or embedded deep within the glandular lumina. One of the keys to successful treatment for these orgainsms is detecting them early so that appropriate therapy can be instituted when they are actively dividing and are more susceptible to therapeutic agents. Therefore, when pertinent clinical information is provided with the biopsy submission, the theriogenologist can be prompted to request special stains that enable the detection of fungal and yeast organisms well before a positive culture result can be obtained.

Embryo transfer programs can benefit from the use of endometrial biopsy. The decision to use a mare as an embryo donor is often made because the endometrial evaluation suggests a poor prognosis to carry a foal to term. In aged, subfertile mares, this is commonly due to chronic degenerative endometrial disease (endometrosis). However regardless of age or parity, a number of embryo donors may have significant subclinical endometritis which could adversely impact embryo recovery and transfer success rates. Having endometrial biopsy findings which substantiates this ahead of time, can alter the therapeutic plan for the donor with regards to the need for pre-breeding or pre-flush treatments, the day of embryo recovery and post-recovery treatment of the embryo (e.g., the number of washes prior to transfer). Contrary to popular belief, donor mares with endometritis can and often do produce embryos that are recovered in lavage effluent that contains considerable cellular debris. However, regardless of how many times an embryo is washed, getting these contaminated embryos to thrive after transfer can be difficult.[9] Therefore, embryo transfer should not be viewed as a substitute for therapies to eliminate endometritis in donor mares.

Screening of all embryo recipients via endometrial biopsy would be ideal, but is often not feasible with large recipient herds. When the number of recipient mares is limited, this practice is strongly encouraged and can allow selection of the best recipient. In order to optimize success, embryos should be transferred into the most hospitable uterine environment possible and the use of endometrial biopsy helps determine this. Transferring an embryo into a recipient with a suboptimal uterine environment is an exercise in futility.

In addition to the GMS and PAS stains for identification of yeast and fungi, a number of other techniques have been described to identify various endometrial pathologies. Trichrome and picrosirius red stains can be useful for identifying and quantifying endometrial fibrosis.[13,14] Techniques such as tissue morphometry, RT-PCR, ELISA and genetic array analysis have been investigated and could provide more quantitative data from endometrial biopsies in the future.[10] While ultrastructural studies using electron microscopy are primarily limited to research applications, other laboratory methods such as immunohistochemistry, ELISA, rt-PCR and *in situ* hybridization techniques are being used to identify pathogens as well as a variety of endometrial responses to inflammation, including antibodies, cytokines and specific growth factors.[10] Refinement of these techniques has significant potential to enhance our ability to more fully interpret endometrial biopsies in the future.

For now, light microscopy using hematoxylin and eosin (H&E) stains continues to be the standard method for histological evaluation of endometrial biopsies. The common pathological changes (e.g., inflammation, periglandular fibrosis, cystic gland distention, lymphatic stasis and endometrial atrophy) can all be diagnosed using this proven method. With this information, therapeutic plans can be directed based upon the endometrial biopsy results, especially when used in conjunction with the mare's clinical findings and history.

References

1. Seaborn E: The oestrous cycle in the mare and some associated phenomena. Anat Rec 1925;30:277-287.

2. Dimock WW, Edwards PR: The pathology and bacteriology of the reproductive organs of mares in relation to sterility. Ky Agr Exp Stat Res Bull 1928; 286.
3. Kenney RM: Prognostic value of endometrial biopsy in the mare. J Reprod Fertil Suppl 1975;23:347-348.
4. Kenney RM. Cyclic and pathologic changes of the mare endometrium as detected by biopsy, with a note on early embryonic death. J Am Vet Med Assoc 1978;172:241-262.
5. Ricketts SW: Endometrial biopsy as a guide to diagnosis of endometrial pathology in the mare. J Reprod Fertil Suppl 1975;23:341-345.
6. Ricketts SW: The technique and clinical application of endometrial biopsy in the mare. Equine Vet J 1975;7:102-108.
7. Ricketts SW, Alonso S: Assessment of the breeding prognosis of mares using paired endometrial biopsy techniques. Equine Vet J 1991;23:185-188.
8. Van Camp SD. Endometrial Biopsy of the mare. Review and update. Vet Clin North Am Equine Pract 1988;4:229-245.
9. Brinsko SP, Blanchard TL, Varner DD, et al.: Manual of equine reproduction, 3rd ed. Maryland Heights (MO): Mosby Inc/Elsevier; 2010.
10. Schlafer DH: Equine endometrial biopsy: enhancement of clinical value by more extensive histopathology and application of new diagnostic techniques? Theriogenology 2007;68:413-422.
11. Kenney RM, Doig PA: Equine endometrial biopsy. In: Morrow D, editor. Current therapy in theriogenology. 2nd ed. Philadelphia: WB Saunders; 1986. p. 723-729.
12. Nielsen JM. Endometritis in the mare: a diagnostic study comparing cultures from swab and biopsy. Theriogenology 2005;64:510-518.
13. Blanchard TL, Garcia MC, Kintner LD, et al: Investigation of the representativeness of a single endometrial sample and the use of trichrome staining to aid in the detection of endometrial fibrosis in the mare. Theriogenology 1987;28:445-450.
14. Ganjam VK, Evans TJ: Equine endometrial fibrosis correlates with 11beta-HSD2, TGF-beta1 and ACE activities. Mol Cell Endocrinol 2006;248:104-108.

Ancillary semen tests for stallions: which ones to use and what do they mean

D.D. Varner, T.L. Blanchard, C.C. Love, S.P. Brinsko
Department of Large Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX

Introduction

Actual fertility trials are the best gauge of a stallion's fertility, be it under natural-cover or artificial-insemination conditions. Nonetheless, use of such trials to assess the intrinsic fertility of a stallion can be complicated by factors such as mare fertility, management issues, time concerns, and associated expenses. Laboratory based examination of semen generally has good predictive value regarding the fertility of a stallion, at least from the perspective of determining if a given stallion will be highly subfertile or have commercially acceptable fertility. Such an examination is generally done for one of four reasons: 1) as part of a pre-purchase examination; 2) as a quality control measure prior to the onset of a breeding season; 3) when reduced fertility is apparent or suspected; or 4) prior to acquisition of some types of fertility-related insurance.

Conventional laboratory tests typically include assessment of total sperm number in ejaculates and assessment of semen quality. Assessment of sperm quality has included light microscopic evaluation of sperm morphologic characteristics and estimation of sperm motility (including percentages of motile and progressively motile sperm; velocity of sperm movement; and longevity of sperm motility following in-vitro storage), as well as presence of blood, urine, or potentially pathogenic bacteria in the semen.[1-5] The value of semen evaluation, as in any type of diagnostic approach, is centered around reliable equipment, good laboratory technique, and personnel with good observational power. Even when these stipulations are thought to be met, the predictive value of the examination has been reported to be limited.[6-21] The same holds true for sperm of other mammalian species.[22-25] As such, more critical and expanded analysis of equine semen might improve the predictive value of the testing process.

The use of the term, ancillary, for describing additional/alternative methods for evaluating semen is quite vague. The term can impiy newly developed assays that impart novel information regarding sperm fertilizing potential; however, it can also denote better ways to measure specific features of semen than are commonly examined by more conventional methods. Both contexts of the term will be addressed in this communication.

Keywords: Stallion, semen evaluation, fertility prediction,

Measurement and prediction of sperm number

Determination of total sperm number in an ejaculate would seem to be a relatively simple procedure, but accurate measurement can be fraught with error. Total sperm number is the product of semen volume and sperm concentration. A first recommendation as an ancillary semen test is to use weight of gel-free semen to determine volume. Semen has a specific gravity (1.0085-1.010) that approaches that of deionized water (1.00);[26] as such, 1 gram $\approx$ 1 mL. Measurement of volume in cups with marked gradations is inaccurate, as is measurement of volume in graduated cylinders that are oversized for the specimen to be measured. For example, use of a 100-mL capacity graduated cylinder would be less accurate than a 25-mL capacity graduated cylinder for measurement of semen volume in the 10-25-mL range. The authors prefer to use a scale with a tare function and a sensitivity of 0.01 g to measure volume of semen. For the measurement, the semen container (prior to insertion of an in-line filter, but with a sterile disposable liner in place, if used) can be weighed prior to attachment to an artificial vagina. The tare function is then activated to enable one to account for the weight of the container; thereby, subsequent placement of the same container with semen on the scale will allow one to account only for the additional weight of the semen. If semen extender is added to the semen container prior to semen collection, this can also be included in the tare activation such that one can accurately determine the volume of gel-free semen in the ejaculate while providing more immediate protection for the semen at the time of semen collection.

Sperm concentration has typically been measured in the clinical arena by hemacytometry or photometric methods.[27] Both of these methods can be inherently inaccurate. Hemocytometer counts have the advantage of directly identifying sperm, but the proclivity for technician mistakes and relatively few sperm in counts can lead to a high margin of error. Photometric methods can also be inaccurate. These methods measure light transmission through a sample and are generally calibrated with hemocytometer counts of sperm. While photometric methods tend to have high repeatability, accuracy is affected negatively by contamination of the ejaculate with debris, urine, or blood. Similarly, sperm concentration cannot be correctly measured when the semen is pre-mixed with an opaque extender, such as a yolk- or milk-based extender. These photometric methods also tend to underestimate sperm concentration when actual sperm concentration is high (i.e., over 300 x 10^6 sperm/mL) and tend to overestimate sperm concentration when actual sperm concentration is relatively low (i.e., less than 100 x 10^6/mL).[28]

Introduction of a fluorescence-based instrument (NucleoCounter® SP-100, Chemometec, A/S, Allerød, Denmark) for the purpose of measuring sperm concentration has, in the view of the authors, revolutionized our procedural methods for analyzing and processing stallion semen. The instrument determines sperm concentration, based on intercalation of the fluorescent dye, propidium iodide, with DNA of sperm after permeabilizing the sperm membranes with a detergent solution to expose the DNA to the dye. The treated sperm emit fluorescence when excited with green light from the fluorescence microscope within the instrument. Studies have demonstrated that this instrument can reliably evaluate sperm concentration, even when semen is pre-mixed in a variety of opaque and non-opaque media.[28] This, in itself, has significant applications in laboratories that process semen for cooled transport or frozen storage. Additionally, the instrument readings compared favorably with hemacytometer and flow cytometer measurements over a large range of sperm concentrations.[28]

Determination of total sperm number in ejaculates is important when proportioning semen for insemination of mares; however, it is also an important part of a breeding soundness evaluation. In this capacity, it may be most important when semen is collected from stallions on a daily basis for several days to determine actual daily sperm output (DSO). This approach can be troublesome for some stallion owners/managers because of the time and costs involved. An alternative method for predicting daily sperm output is to obtain accurate measurements of testicular dimensions and calculate total testicular volume.[29] Testicular volume is highly correlated with daily sperm output in stallions, and provides a more accurate reflection of DSO than does measurement of total scrotal width.[29,30] The formula for testicular volume is as follows: Testicular volume (TV) = $4/3\pi abc$, where a=height/2, b=width/2, c=length/2, and measurements are made in cm; therefore, TV = 0.52 (height x width x length). Predicted DSO is determined as follows: Predicted DSO ($x10^9$) = 0.024x – 0.76, where x=total testicular volume.[29] If spermatogenic efficiency is normal, as would be expected if sperm morphologic and motility features are normal, then predicted daily sperm output generally approximates actual DSO. Our laboratory also compares actual DSO to predicted DSO in some stallions with reduced fertility to determine spermatogenic efficiency ([Actual DSO/Predicted DSO] x 100).[31,32] In aging breeding stallions with deteriorating testicular function, a decline in spermatogenic efficiency will oftentimes precede declines in testicular size or semen quality.[32] This can also be the case in some stallions with a transitory decline in fertility. As a case in point, Table 1 provides information on predicted and actual daily sperm output, as well as measures of sperm motility and sperm morphology in a middle-aged Thoroughbred stallion with a history of high fertility in previous years (> 90% seasonal pregnancy rate when covering over 150 mares per season with a spermatogenic efficiency that approached 100%). The stallion underwent general anesthesia to correct a nephrosplenic entrapment (by rolling) the November prior to the breeding season and was treated medically for iliac thrombosis in January prior to the breeding season. Per-cycle pregnancy rate for February-April was 22% (31/141), and per-cycle pregnancy rates for May and June were 1.5% (1/67) and 5% (2/41) respectively.

As shown in Table 1, minimal changes were detected in testicular size and sperm quality (as judged by percentages of total sperm motility, progressive sperm motility, and morphologically normal sperm) during the examination period. Circulating hormonal values and sperm chromatin values were within normal limits during this time. Spermatogenic efficiency was low in April, dropped precipitously

in May, began to rebound in June, and increased markedly by November. In mid-November, 7 mares were bred by natural cover and 5 became pregnant (71% per-cycle pregnancy rate). The stallion had normal fertility (>90% seasonal pregnancy rate) the following commercial breeding season. With this clinical case, spermatogenic efficiency appeared to be the most sensitive indicator of fertility.

Measures of sperm quality

Sperm quality is considered to be important to fertility of males, including stallions, when fertilization occurs under *in vivo* conditions. Nonetheless, no single measure, or combination of measures, for semen quality has an exact correlation to fertility. In fact, the strength of the relationship for traditional measures, such as sperm motility and sperm morphology, is relatively low, with these measures singularly accounting for less than 50% of variability in pregnancy rate per cycle for groups of commercial breeding stallions.[33-35] Relatively low correlations can be attributed, in part, to mare and management factors that could confound one's ability to use laboratory measurements to predict intrinsic stallion fertility; however, if pregnancy data are corrected to help remove such potential confounders (by measuring first-cycle pregnancy rate, i.e., the number of mares pregnant in their first breeding cycle divided by the number of mares pregnant at the end of the breeding season), singular laboratory measures of sperm quality still do not account for the majority of variation stallion fertility.[35] Given this information, it is logical to consider development/implementation of a battery of laboratory assays that could more clearly define fertilizing potential of stallion sperm. Fertilization *in vivo* requires an array of highly coordinated cellular and molecular events so it would seem intuitive to evaluate various sperm compartments and characteristics in an effort to determine if a sperm population has the attributes necessary for efficient fertilization. While this approach has been considered by others in years past,[36] a decade later we still do not have the "magical formula" for explicitly predicting the fertility of stallions. That said, it remains important to pursue development of laboratory assays that can be useful for more effectively predicting stallion fertility.

Sperm motility

Measurement of sperm motility is likely to remain a hallmark of semen evaluation, and it is relatively easy to perform. Evaluation of sperm motility in both raw and extended forms is considered to be a fundamental laboratory test for assessing the fertilizing capacity of sperm in an ejaculate.[1] Dissimilarity in scores among examiners is inevitable when the evaluation is done subjectively, but this variation can be minimized when a high-quality microscope with phase contrast optics and a warming stage is used. Evaluation of raw (undiluted or neat) ejaculated semen gives one an idea of how well sperm perform in their natural milieu, i.e., seminal plasma. Determining motility in the raw semen can be hampered by higher sperm concentrations and sperm agglutination to the cover glass, making it difficult for the evaluator to discern individual motility patterns. To overcome this limitation, an aliquot of semen can also be appropriately diluted (e.g., to 25-30 x 10^6 sperm/ml) in a good quality semen extender that is free of microscopic debris. The extender may slightly alter motility pattern, usually by increasing the sperm progressivity and velocity measures. After initial extension, a high percentage of sperm may exhibit a circular motility pattern; however, this behavior oftentimes resolves following 5-10 minutes of exposure time in warmed (37°C) extender. A downside of sperm motility as a laboratory measure of sperm function is that sperm motility is susceptible to environmental conditions (e.g., excessive heat or cold, lubricants, light, disinfectants, and osmolarity/pH of semen extender), so it is necessary to protect the semen from injurious agents or conditions prior to analysis. Estimations of the percentages of motile and progressively motile sperm are generally determined, in addition to an estimation of sperm velocity (based on an arbitrary scale of 0 [stationary] to 4 [fast]). Subjective assessment of motility is generally quite acceptable, provided personnel are experienced in analysis of sperm motility.

Several different techniques and instruments have been developed in an effort achieve an objective (i.e., unbiased) evaluation of sperm motility; however, these methods (e.g., time-lapse photomicrography, frame-by-frame playback videomicrography, spectophotometry, or computerized analysis) are generally considered to be too tedious or expensive for routine use. Computerized systems

are currently in place in many reference laboratories with the intent to objectively assess motion characteristics of sperm. Despite the commercial availability of various generations of computer-assisted sperm analysis (CASA) systems for over two decades, their presence has not provided the definitive assay for measuring sperm fertilizing potential.[37] Such an expectation, however, is unrealistic given the numerous independent sperm attributes that are required for a spermatozoon to possess fertilization competence. What these systems do provide is the prospect of objective measurement and protocol standardization. These instruments permit customized selection of various features, including frequency and length of frame capture; threshold demarcations for sperm motion, progressivity of motion, and velocity measures; and gating freedom for both size and luminosity representative of sperm heads, as a means to maximize capture of sperm while minimizing capture of non-sperm material in the sample of interest. Such manipulations are important for improving measurement accuracy and repeatability within a given laboratory, but make it virtually impossible for reliable comparisons among laboratories or among different CASA brands.[38,39] Computerized analysis of sperm is primarily reserved for the research setting, where standardization, accuracy, and precision are a prerequisite to measurement of experimental end points. A distinct value of CASA instruments in the commercial environment (at a veterinary hospital or an equine breeding operation) is the ability to garner objective results for a variety of motility variables. Confusion arises, however regarding the relationship of the myriad of obtainable CASA variables to fertility of the sample. As an example, we conducted a fertility trial with a subfertile stallion whose semen was subjected to density-gradient centrifugation in an effort to improve semen quality prior to insemination. Values for percent total motility, percent progressive motility, and mean curvilinear velocity prior to, and after, semen processing for the subfertile stallion and a fertile control stallion are listed in Table 2.

Based on these results, it would appear that semen treatment for the subfertile stallion yielded a sperm population with quality similar to, or exceeding (based on velocity values), that of the fertile control stallion. Nonetheless, when fertile mares were inseminated hysteroscopically with 20×10^6 progressively motile sperm (100 µl-volume), the resulting pregnancy rates were 15/20 (75%) for the fertile stallion, as compared to 7/20 (35%) for the subfertile stallion ($P<0.05$). This demonstrates that sperm motility does not provide absolute discrimination power, again emphasizing that sperm attributes other than motility play critical roles in sperm fertilizing ability. Others have reported that sperm populations possess several clusters (subpopulations) of sperm with specific sperm motion characteristics;[40] however, the specific relationships of these semen characteristics to fertility have not been described. Some CASA systems can be outfitted with fluorescence optics, and the predictive value of CASA might be improved by incorporation of fluorescent dyes in the media such that motility variables can be segregated by presence or absence of plasma membrane intactness (integrity).[41] Further studies are required to determine if the predictive value of sperm motility can be improved by this approach. This topic is addressed further below under the section regarding plasma membrane integrity.

Sperm morphology

The morphology of sperm is typically examined with a light microscope at 1000X magnification. Standard bright-field microscope optics can be used to examine air-dried semen smears, provided appropriate stains are used in slide preparation. Specific sperm stains include those developed by Williams[42] and Casarett.[43] General-purpose cellular stains (*e.g.*, Wright's, Giemsa, hematoxylin-eosin) also have been used to accent both germinal and somatic cells in semen smears. A recent study described the use of Diff Quik (a commercially available modification of the Wright-Giemsa formula and containing triarymethane, xanthene and thiazine dyes) for morphologic assessment of stallion sperm.[44] Background stains (*e.g.*, eosin-nigrosin, India ink) probably are the most widely used stains because of their ease of application. In the authors' view, visualization of the structural detail of sperm can be greatly enhanced by fixing the cells in buffered formol saline or a similar fixative, then viewing the unstained cells as a wet mount with either phase-contrast or, preferably, differential interference contrast (DIC) microscopy.[1] In addition, the incidence of artifactual changes is reduced in comparison with stained smears.

In our laboratory, at least 100 sperm per specimen are evaluated for evidence of morphological defects. The type and incidence of each defect is recorded. Abnormalities in sperm morphology traditionally have been classified as primary, secondary or tertiary. Primary abnormalities are considered to be associated with a defect in spermatogenesis and, therefore, are of testicular origin. Secondary abnormalities are created in the excurrent duct system. Tertiary abnormalities, as opposed to the previous two types, develop in vitro as a result of improper semen collection or handling procedures. The current trend is to record the percentages of specific morphologic defects, e.g., abnormal heads, knobbed acrosomes, proximal protoplasmic droplets, swollen midpieces, coiled tails, etc. The authors consider this method of classification to be superior to the traditional system because it reveals more specific information regarding a population of sperm, while avoiding erroneous assumptions about the origin of these defects. The origin of some sperm morphologic defects is unknown. Additionally, some morphologic abnormalities like detached heads can be primary, secondary or tertiary in nature, thereby introducing the possibility of error when using the traditional classification system exclusively.

In one study, the percentage of morphologically normal sperm had a relatively high correlation to percentages of total and progressive sperm motility and to measures of sperm velocity. Similarly, the percentages of several sperm morphologic abnormalities (i.e., midpiece abnormalities, detached heads, bent tails, coiled tails, and premature germ cells) were negatively correlated with percentage of morphologically normal sperm.[35] Such correlations cannot be considered exact and can vary among individual stallions and ejaculates. For instance, some semen samples can possess good sperm motility, yet have a relatively high incidence of certain sperm morphologic abnormalities, such as proximal or distal cytoplasmic droplets. Generally speaking, the percentage of morphologically normal sperm in a semen sample is similar to the percentage of progressively motile sperm in fresh extended semen. If sperm motility is low and the percentage of morphologically normal sperm is high, it suggests that laboratory errors (mishandling of semen) occurred which led to a lowering of sperm motility. One cannot discount, however, a potentially negative effect of seminal plasma on sperm motion characteristics (discussed below under the section on seminal plasma toxicity) or abnormalities of sperm ultrastructure that are not perceptible when using light microscopy. A distinct advantage of sperm morphology over sperm motility for judgment of sperm quality is that sperm morphological features are less disrupted by environmental disturbances than are measures of sperm motility.

Sperm structure is undoubtedly related to fertility; however, the impact of specific sperm morphologic features on fertility remains unclear. For instance, investigators have reported that the incidence of proximal cytoplasmic droplets can negatively impact fertility,[33] has a questionable impact on fertility,[35] or has no significant impact on fertility.[8] Significant intra-stallion variation in sperm morphology typically occurs in commercial breeding stallions without impacting fertility;[8] as such, isolated examinations of sperm morphology may not be as reliable an indicator of fertility as would more frequent sperm morphologic examinations. Investigators are generally in agreement that the percentage of morphologically normal sperm is positively correlated with fertility.[8,33-35] Increasing percentages of defects considered to be major, i.e., abnormal heads or abnormally shaped midpieces, are associated with a corresponding reduction in fertility.[8,35] Increasing percentages of coiled tails and premature germ cells may also be associated with reduced fertility.[8] Conversely, percentages of bent tails and distal droplets do not appear to have any predictive value for fertility.[8,33,35] If the morphologically abnormal sperm do not exert a direct negative influence on normal sperm, it is possible that the total number of morphologically normal sperm in ejaculates may provide more information regarding the fertility of a stallion than the percentage or absolute number of morphologically abnormal sperm.

Transmission electron microscopy can provide striking detail of sperm ultrastructure and other constituents of semen. Others have recommended this technique as a means of more critically assessing sperm morphology at the ultrastructural level and identifying other components of semen that may not be detectable with light microscopy.[45] The authors contend that insufficient information can be gleaned from transmission electron microscopy of sperm on a routine basis to rationalize the additional time and expense associated with this method of evaluation. Nonetheless, transmission electron microscopy may be well justified with selected cases.

Plasma membrane integrity (intactness)

The semi-permeable plasma membrane, which is composed of a wide assortment of lipids, proteins, and carbohydrates, envelops the entire spermatozoon. This structure is vital to regulation of sperm functions by establishing ion gradients, facilitating cytosolic entry of larger molecules, and orchestrating various cell-signaling events, to name a few. Thus, assessment of its integrity would seem an important component of a semen evaluation. To this end, a variety of laboratory procedures have been used to evaluate the integrity of the plasma membrane. One method is to evaluate the ability of sperm to exclude extracellular dyes, such as eosin Y, which are nonpermeable when the membrane is intact.[46] Another approach is to expose sperm to hypotonic media (50-100 mOsm range) to test their osmoregulatory function (termed the hypo-osmotic swelling test; HOST).[47-49] With this assay, membrane intact sperm theoretically permit excessive water entry into the cytosol, resulting in a variety of morphological changes in the flagellum associated with the cytosolic swelling. Conversely, osmoregulation-incompetent sperm will not experience noticeable changes in flagellar shape. While these tests can serve an adjunctive role in semen evaluation, they are not widely applied in the clinical setting because of the potential for misinterpretation. For instance, the incidence of sperm with a bent flagellum prior to HOST will also confound the interpretation of results following exposure of a sperm population to hypo-osmotic media.

In recent years, fluorescent dyes have generally replaced non-fluorescent dyes for evaluation of plasma membrane integrity. A broad array of membrane-impermeable fluorescent dyes is available commercially and can be used to test membrane intactness. Examples include the DNA dyes, propidium iodide, bis-benzimide (Hoechst 33258), YO-PRO®-1, TOTO®-1, and ethidium homodimer-1. As an alternative, sperm can be bathed in cell-permeable probes that become hydrolyzed to form membrane-impermeant fluorescent products in the cytosol. Examples include carboxyfluorescein diacetate (hydrolyzed by nonspecific cytosolic esterases to form carboxyfluorescein); calcein AM or dihydrocalcein AM (hydrolyzed by esterases to form calcein, which, in turn, forms fluorescent complexes with Ca^{2+}, and other metals) and SYBR®-14 (deacylated in the cytosol, with the resulting product expressing strong fluorescence when complexed with nucleic acids). Of interest, staining of porcine sperm with SYBR®-14, which complexes with DNA, does not affect their ability to fertilize oocytes.[50] Certain membrane-impermeable and membrane-permeable dyes can be combined in solution prior to sperm exposure in an effort to provide a more accurate reflection of membrane integrity. For instance, a combination of SYBR®-14 and propidium iodide yields three populations of stained sperm: 1) membrane-intact, SYBR®-14-stained cells (green); 2) membrane-damaged, propidium-iodide-stained cells (red), and 3) moribund cells (double-stained).[51,52] Some fluorescent plasma-membrane dyes can also be combined with certain mitochondrial dyes or acrosomal dyes to provide more thorough compartmental coverage in the assay.[53-59] The literature reports triple-stain fluorescent techniques for use with stallion sperm: propidium iodide/SYBR®-14/JC1,[54] and propidium iodide/FITC-PNA (an acrosomal lectin probe)/carboxy-SNARF-1(an intracellular pH indicator).[55,60] Although images can be ascertained with the various fluorophores described above by using fluorescence microscopy, flow cytometry is typically applied because of the high through-put (i.e., many more sperm are counted) and objectivity associated with this approach.[59,61-64] Utilization of a fluorescence microplate reader assay is also reported for use with JC-1.[65]

The relationship between plasma membrane integrity and sperm motility remains unclear. In one study, the percentage of motile stallion sperm (based on computerized motility analysis) was highly correlated (r=0.98) with the percentage of sperm with intact plasma membranes, based on staining with SYBR®-14 and propidium iodide.[54] Others have reported that sperm motility declines at a more rapid rate than plasma membrane integrity in extended, cool-stored semen, and that separation between these endpoints is more pronounced when the extended semen contains a high percentage of seminal plasma.[66,67] Interestingly, daily centrifugation and resuspension of sperm in fresh extender containing only 10% seminal plasma aids in protecting sperm from declines in sperm motility and membrane intactness following cooled storage for up to 96 h.[67] From these studies, it would appear that sperm

motility is more negatively impacted by cooled storage than is plasma membrane integrity. Recently, Kiser et al. reported that percent total sperm motility was significantly lower in extended semen stored for four days in 50% seminal plasma (6%), as compared to 10% seminal plasma (55%) from a single stallion; however, percent viable sperm was similar between the two treatment groups (75 and 74%, respectively). One-cycle pregnancy rates for mares bred with stored semen were similar between semen-treatment groups (45% [5/11] and 58% [7/12], respectively)[68] These results suggest that sperm plasma membrane viability may be a better predictor of fertility than sperm motility for predicting the fertility of cool-stored semen.

Sperm chromatin quality

Assessment of sperm chromatin quality addresses a compartment of sperm that may not be assessed by light microscopic evaluation of sperm motility or sperm morphology, and such assays are routinely incorporated into sperm evaluations in some laboratories. One of these tests is the sperm chromatin structure assay (SCSA). This assay, introduced by Evenson in 1980,[69] has been applied to sperm from a number of species, including horses.[70-72] This assay has been shown to be a useful predictor of fertility in stallions.[71] The SCSA is a flow cytometric procedure that utilizes the metachromatic fluorochrome, acridine orange, and tests the denaturability of sperm chromatin challenged with acid treatment. The literature contains variable results regarding the relationship of stallion sperm chromatin denaturation to the extent of disulfide bonding within and between protamine molecules;[73,74] however, chromatin susceptibility to denaturation is correlated with the level of actual DNA strand breaks.[72] The DNA strand breaks can be associated with a myriad of factors, including idiopathic apoptosis, oxidative stress, heat stress, radiation injury, or protamine deficiency,[74-81] and may involve double-stranded or single stranded DNA fragmentation or oxidized nucleosides.[75-82] Such lesions could create genetically defective sperm, leading to germ-line mutations. Interestingly, sperm affected by such damage may appear to be normal, based on laboratory parameters such as sperm motility and membrane integrity, but may induce post-fertilization embryonic failure.[83] Owing to the highly condensed nature of the sperm chromatin, mature sperm are known to be transcriptionally inactive,[84] so it is logical that DNA damage might not be expressed until mitosis occurs at the time of spermatozoon-oocyte fusion. This becomes quite important clinically as it represents a potential noncompensible defect, i.e., affected sperm in an ejaculate may not be impaired for fertilization, so increasing the insemination number may not increase pregnancy rate, but may contribute to early embryoic death. Ejaculated sperm are known to retain a cohort of cytoplasmic mRNAs with translational ability,[84,85] so it is also possible that fragmentation of mRNA could have a negative impact on some other sperm functions leading to reduced fertilization potential.

Assays other than the SCSA are available to measure sperm DNA fragmentation/chromatin disruption, including a TdT-mediated-dUTP nick end labeling (TUNEL) assay, an in-situ nick translation (NT) assay, a sperm chromatin dispersion (SCD) assay, and an electrophoresis-based Comet Assay.[75,76,86-91] While such assays have been applied only on a limited basis in stallions,[92-94] they are commonly used in the human field. An immunofluorescence assay has also been developed for evaluation of human sperm protamine levels,[77] and a similar assay for equine sperm could have diagnostic value.

Acrosomal responsiveness

Our laboratory has identified a subset of highly subfertile Thoroughbred stallions whose sperm acrosomes do not undergo the acrosome reaction effectively when the sperm are exposed to a potent inducer of this reaction.[95] The underlying mechanism of this defect remains unclear; however, further studies have revealed that the cholesterol-to-phospholipid ratio in sperm of these stallions is significantly higher than that of fertile control stallions.[96] Recently, a susceptibility locus for impaired acrosome reaction (FKBP6) has been discovered in this group of stallions and the frequency of the genotype for impaired acrosomal reaction was estimated to be 7% in Thoroughbreds.[97] Conventional tests are unable to predict the fertility of stallions with this form of acrosomal dysfunction, because these stallions present with normal sperm morphology and motility, as well as normal chromatin quality (based on SCSA

testing) and normal acrosomal structure (based on fluorescence microscopy and transmission electron microscopy). Our laboratory routinely performs an acrosomal responsiveness assay for stallions suspected of having this condition. This assay is directed at testing the functionality of the sperm acrosome, i.e., its ability to acrosome react when challenged with a potent inducer of the event, the Ca^{2+} ionophore, A23187.[95,98] While the acrosome reaction can be identified readily using transmission electron microscopy, fluorescence-based microscopy or flow cytometry using lectin agglutinin isolated from the pea, *Pisum sativum*, or the peanut, *Arachis hypogaea*, can also be used successfully for this purpose.[99-101] Although the more popular fluoresceinated lectin markers have generally replaced chlortetracycline-based assays for detecting the acrosome reaction, the latter assay remains useful because of its ability to gage capacitation, as well as the acrosome reaction.[102-104] Others report that acrosomal status can be assessed by bright-field microscopy using Commassie blue stain.[105,106]

Meyers and co-workers first reported that the acrosomes of subfertile stallions with poor sperm motility did not react readily in response to progesterone exposure.[107] Fertile stallions averaged a 17% acrosomal reaction rate following five hours of incubation in capacitating conditions followed by exposure to progesterone, while subfertile stallions averaged only a 6% response rate. This study indicated that progesterone was capable of stimulating the acrosome reaction in equine sperm exposed to capacitating conditions, and the response in subfertile stallions was reduced. Others have reported that the plasma membrane of stallion sperm contains progesterone receptors, and indicate that this may be a pathway for induction of the acrosome reaction.[108] In this regard, the percentage of sperm with exposed progesterone receptors has been shown to have a high correlation with fertility of stallions ($r=0.70$).[109]

Other assays for sperm quality

Considerable effort has been directed toward identification of biochemical markers of sperm function that might aid in laboratory-based prediction of fertility by targeting specific subcellular compartments or domains. Many of these methods have been devised for use with non-equids, but several have been proposed for potential use with stallion sperm. Although the value of such tests requires further scrutiny and standardization, Table 3 provides examples of assays that may one day prove valuable as diagnostic tools:

Seminal plasma toxicity

Seminal plasma represents the non-sperm portion of semen, with contributions from the rete testis, epididymis, deferent ducts, and the accessory genital glands. Seminal plasma is of very complex composition, containing a rich assortment of both organic and inorganic constituents.[195-197] While it is considered "an essential attribute" to sperm,[196] with functions ranging from sperm transport in the female, to modulation of uterine inflammation, oxidative injury, and sperm capacitation, to nutritive support and other protective roles, seminal plasma, as it exists in ejaculated semen, is not essential to fertilization. This statement can be supported by the fact that mares (and females of other species) can become pregnant when inseminated with epididymal sperm that have not been exposed to contributions from the accessory genital glands. Possibly, the contributions of seminal plasma which arise from the rete testis and epididymis are essential to fertilization. Under natural conditions, sperm are exposed to a high concentration of ejaculated seminal plasma for a relatively short period of time. Studies have demonstrated that seminal plasma can adversely affect some properties of sperm following prolonged exposure at elevated concentrations.[67,198-201] Significant inter-stallion variation also exists regarding seminal plasma effects on semen quality following either short-term or long-term exposure.[202]

Some stallions possess seminal plasma that appears "toxic" to their sperm, as evidenced by a reduction in sperm motility percentage or sperm velocity, as compared to sperm from the same ejaculate following exposure to seminal plasma from another stallion.[203] Other stallions ejaculate sperm that are negatively impacted by their own seminal plasma or seminal plasma from other fertile control stallions.[31] Stallions with normal semen quality in fresh ejaculates, but rapidly reduced semen quality following cooled storage in extender can be subjected to testing for susceptibility to seminal plasma. Extended semen of the stallion in question is subjected to centrifugation, followed by removal of seminal plasma,

and resuspension of sperm in homologous seminal plasma, seminal plasma of a fertile control stallion, or extender free of seminal plasma. While most commonly used semen extenders do no support good sperm motility if no seminal plasma is present, milk-based extender containing modified Tyrodes medium can be used effectively for extended semen where no seminal plasma is present.[200,204] Following such experimentation, one can determine if a stallion's own seminal plasma is problematic to sperm function, or if all seminal plasma imparts a negative effect of sperm function. Management strategies can then be implemented to improve the reproductive performance of these stallions.[31,203]

Concluding remarks

Although a plethora of scientific information surrounds sperm structure and function, many unresolved issues remain, even with human and rodent sperm where the largest body of information has been assimilated. Only when we know precisely the specific molecular interactions required for attaining full sperm fertilizing potential, including the spatial and temporal changes, energetics, and gaseous environment involved, will we be able to reliably manipulate sperm to meet the growing needs within the equine breeding industry. While these might appear to be lofty goals, a more absolute understanding of sperm structure and function would certainly take a lot of the "guess work" out of current approaches to analysis and manipulation of equine sperm. Attempts to gain a better understanding of equine sperm solely from extrapolation of data acquired from other mammalian species are likely destined to failure because of the well-known species differences in sperm attributes and physiology. Nonetheless, much information derived from other species may have relevance to equids, and should be investigated for applicability. Examples include identification of candidate genes for specific sperm traits, targeted mutation of genes for specific proteins to study the resulting effect on reproductive function, and use of gene silencing agents for regulatable ablation of gene function. Incorporation of molecular techniques would appear to be the key to elucidation of mechanisms that control sperm function in stallions.

Table 1. Measures of testicular volume, predicted daily sperm output, actual daily sperm output, spermatogenic efficiency, total sperm motility, progressive sperm motility, and morphologically normal sperm in a stallion with a transient, but profound, reduction in fertility.

Parameter	27 Apr	19 May	24 Jun	24 Sep	15 Nov
Total testicular volume (mL)	435	459	471	480	427
Predicted daily sperm output (x 10^9)	9.7	10.3	10.5	10.8	9.5
Actual daily sperm output (x 10^9)	4.6	2.1	5.0	7.6	7.5
Spermatogenic efficiency (%)	47	20	48	70	79
Total sperm motility (%)/	65	55	65	60	65
Progressive sperm motility (%)	40	40	30	45	40
Morphologically normal sperm (%)	56	49	42	49	47

Table 2. Evaluation of sperm motility values from a one fertile and one subfertile stallion before, and after, subjecting the semen to density-gradient centrifugation.

Stallion	Semen treatment	Total sperm motility (%)	Progressive sperm motility (%)	Curvilinear velocity (μm/s)
Fertile	Before	80	63	205
Fertile	After	91	78	209
Subfertile	Before	63	48	251
Subfertile	After	90	79	259

Table 3. Examples of biochemical markers that have been used to assess sperm function in various mammalian species.

Biochemical marker	Potential value of test
Acrosin/amidase activity[110-112]	Acrosome integrity
SNARE proteins[113,114]	Acrosome reaction
Caspases[115-118]	Apoptosis
Heparin-binding proteins[119,120]	Capacitation
Protein phosphotyrosine activity[121-123]	Capacitation
Superoxide anion[124]	Capacitation
Chromomycin A_3[125-128]	Chromatin packing
Phospholipase $C\zeta$[129-131]	Oocyte activation
C_{11}-BODIPY(581/591)[132-137]	Oxidative injury
Malondialdehyde[138-145]	Oxidative injury
Glutathione peroxidase[146-150]	Oxidative injury
Reactive oxygen species[23,145,149,151-158]	Oxidative injury
Lactate dehydrogenase[159-162]	Sperm motility
Creatine kinase/heat shock protein A2[163-166]	Sperm maturity
CRISP proteins[119,167-172]	Sperm-oocyte binding
SP20/hyaluronidase[173-175]	Sperm-oocyte interaction
AWN spermadhesion protein[119,176-178]	Sperm-zona binding
P34H protein[179-182]	Sperm-zona binding
Zonadhesin[183-190]	Sperm-zona binding
SP22 protein[191-194]	Sperm fertility

References

1. Kenney RM, Hurtgen J, Pierson R, et al: Theriogenology and the equine. Part II. The stallion (Society for Theriogenology manual for clinical fertility evaluation of the stallion). Hastings (NE): Society For Theriogenology; 1983.
2. Blanchard TL, Varner DD, Schumacher J, et al: Examination of the stallion for breeding soundness. In: Blanchard TL, Varner DD, Schumacher J, et al., editors. Manual of equine reproduction. St. Louis: Mosby; 2003. p. 143-164.
3. Jasko DJ: Evaluation of stallion semen. Vet Clin North Am Equine Prac1992;8:129-148.
4. Varner DD, Schumacher J, Blanchard TL, et al: Diseases and management of breeding stallions. Goleta (CA): American Veterinary Publications; 1991.
5. Pickett BW: Reproductive evaluation of the stallion. In: McKinnon AM, Voss JL, editors. Equine reproduction. Philadelphia: Lea and Febiger; 1993. p. 755-768.
6. Jasko DJ, Little TV, Lein DH, et al: Determination of stallion semen quality and its relationship with fertility. J Reprod Fertil 1991;Suppl 44:649-650.
7. Jasko DJ, Little TV, Lein DH, et al: Comparison of spermatozoal movement and semen characteristics with fertility in stallions: 64 cases (1987-1988). J Am Vet Med Assoc 1992;200:979-985.
8. Love CC, Varner DD, Thompson JA: Intra- and inter-stallion variation in sperm morphology and their relationship with fertility. J Reprod Fertil 2000;Suppl 56:93-100.
9. Kenney RM, Kingston RS, Rajamannon AH, et al: Stallion semen characteristics for predicting fertility. Proc Annu Conv Am Assoc Equine Pract 1971. p.53-67.
10. Colenbrander B, Puyk H, Zandee AR, et al: Evaluation of the stallion for breeding. Acta Vet Scand 1992;Suppl 88:29-37.
11. Amann RP: Can the fertility potential of a seminal sample be predicted accurately? J Androl 1989;10:89-98.
12. Bielanski W, Kaczmarski F: Morphology of spermatozoa in semen from stallions of normal fertility. J Reprod Fertil 1979;Suppl 27:39-45.
13. Bielanski W, Dudek E, Bittmar A, et al: Some characteristics of common abnormal forms of spermatozoa in highly fertile stallions. J Reprod Fertil 1982;Suppl 32:21-26.
14. David JSE: Semen examination for fertility potential in stallions. J Reprod Fertil 1982;Suppl 32:635.
15. Voss JL, Pickett BW, Loomis PR: The relationship between seminal characteristics and fertility in thoroughbred stallions. J Reprod Fertil 1982;Suppl 32:635-636.
16. Dott HM: Morphology of stallion spermatozoa. J Reprod Fertil 1975;Suppl 23:41-46.

17. Rousset H, Chanteloube P, Magistrini M, et al: Assessment of fertility and semen evaluations of stallions. J Reprod Fertil 1987;Suppl 35:25-31.
18. Dowsett KF, Pattie WA: Characteristics and fertility of stallion semen. J Reprod Fertil 1982;Suppl 32:1-8.
19. Voss JL, Pickett BW, Squires EL: Stallion spermaatozoal morphology and motility and their relationships to fertility. J Am Vet Med Assoc 1981;178:287-289.
20. Clement F, Ladonnet Y, Magistrini M: Sperm morphology and fertility. Anim Reprod Sci 2001;68:362-363.
21. Kenney RM, Evenson DP, Garcia MC, et al: Relationships between sperm chromatin structure, motility, and morphology of ejaculated sperm, and seasonal pregnancy rate. Biol Reprod Mono 1 1995;6:647-653.
22. Wang C, Chan SYW, Ng M, et al: Diagnostic value of sperm function tests and routine semen analyses in fertile and infertile men. J Androl 1988;9:384-389.
23. Aitken RJ: Sperm function tests and fertility. Int J Androl 2006;29:69-75.
24. Rodriguez-Martinez H: Can we increase the estimative value of semen assessment? Reprod Domest Anim 2006;41:2-10.
25. Graham JK, Moce E: Fertility evaluation of frozen/thawed semen. Theriogenology 2005;64:492-504.
26. Guerreiro RT: Specific gravity of semen of various domestic animals. Rev Med Vet 1940;35:272-274.
27. Love CC: Measurement of concentration and viability in stallion sperm. J Equine Vet Sci 2012;32:464-466.
28. Comerford K: Validation of a commercially available fluorescence-based instrument to evaluate stallion spermatozoal concentration and comparison to photometric systems [thesis]. College Station (TX): Texas A&M University; 2009. (http://repository.tamu.edu/bitstream/handle/1969.1/ETD-TAMU-2009-05-261/1_Comerford.pdf).
29. Love CC, Garcia MC, Riera FR, et al: Use of testicular volume to predict daily sperm output in the stallion. Proc Annu Conv Am Assoc Eqiuine Pract; 1990. p. 12-21.
30. Gebauer MR, Pickett BW, Voss JL, et al: Reproductive physiology of the stallion: daily sperm output and testicular measurements. J Am Vet Med Assoc 1974:165:711-713.
31. Varner DD, Love CC, Brinsko SP, et al: Semen processing for the subfertile stallion. J Equine Vet Sci 2008;28:677-685.
32. Blanchard TL, Varner DD, Love CC, et al: Management options for the aged breeding stallion with declining testicular function. J Equine Vet Sci 2012;32:430-435.
33. Jasko DJ, Lein DH, Foote RH: Stallion spermatozoal morphology and its relationship to spermatozoal motility and fertility. Proc Annu Conv Am Assoc Equine Pract; 1992. p. 211-221.
34. Jasko DJ, Little TV, Lein DH, et al: Comparison of spermatozoal movement and semen characteristics with fertility in stallions: 64 cases (1987-1988). J Am Vet Med Assoc 1992;200:979-985.
35. Love CC: Relationship between sperm motility, morphology and the fertility of stallions. Theriogenology 2011;76:547-557.
36. Colenbrander B, Gadella BM, Stout TA: The predictive value of semen analysis in the evaluation of stallion fertility. Reprod Domest Anim 2003;38:305-311.
37. Amann RP, Katz DF: Reflections on CASA after 25 years. J Androl 2004;25:317-325.
38. Jasko DJ, Lein DH, Foote RH: A comparison of two computer-automated semen analysis instruments for the evaluation of sperm motion characteristics in the stallion. J Androl 1990;11:453-459.
39. Hoogewijs MK, Govaere JL, Rijsselaere T, et al: Influence of technical settings on CASA motility parameters of frozen thawed stallion semen. Proc Annu Conv Am Assoc Equine Pract; 2009. p. 336-337.
40. Quintero-Moreno A, Miró J, Teresa Rigau A, et al: Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. Theriogenology 2003;59:1973-1990.
41. Wessel MT, Althouse GC: Validation of an objective approach for simultaneous assessment of viability and motility of fresh and cooled equine spermatozoa. Anim Reprod Sci 2006;94:21-22.
42. Williams WW: Cytology of the human spermatozoon. Fertil Steril 1950;1:199-215.
43. Casarett GW: A one-solution stain for spermatozoa. Stain Technol 1953;28:125-127.
44. Pozor MA, Zambrano GL, Runcan E, et al: Usefulness of Dif Quick in evaluating sperm morphology in stallions. Proc Annu Conv Am Assoc Equine Pract; 2012. p. 506-510.
45. Veeramachaneni DNR: Spermatozoal morphology. In: McKinnon AO, Squires EL, Vaala WE, et al, editors. Equine reproduction, 2nd ed. Ames (IA): Wiley-Blackwell; 2011. p. 1297-1307.
46. Blom E. A simple rapid staining method for the differentiation between live and dead sperm cells by means of eosin and nigrosin. Nord Vet Med 1950;2:58-61.
47. Neild D, Chaves G, Flores M, et al: Hyoposmotic test in equine spermatozoa. Theriogenology 1999;51:721-727.
48. Neild DM, Chaves MG, Flores M, et al: The HOS test and its relationship to fertility in the stallion. Andrologia 2000;32:351-355.
49. Nie GJ, Wenzel JG: adaptation of the hypoosmotic swelling test to assess functional integrity of stallion spermatozoal plasma membranes. Theriogenology 2001;55:1005-1018.
50. Garner DL, Dobrinsky JR, Welch GR, et al: Porcine sperm viability, oocyte fertilization and embryo development after staining spermatozoa with SYBR-14. Theriogenology 1996;45:1103-1113.
51. Garner DL, Johnson LA: Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol Reprod 1995;53:276-284.
52. Garner DL, Thomas CA, Allen CH: Effect of semen dilution on bovine sperm viability as determined by dual-DNA staining and flow cytometry. J Androl 1997;18:324-331.

53. Garner DL, Thomas CA: Organelle-specific probe JC-1 identifies membrane potential differences in the mitochondrial function of bovine sperm. Mol Reprod Dev 1999;53:222-229.
54. Love CC, Thompson JA, Brinsko SP, et al: Relationship between stallion sperm motility and viability as detected by two fluorescence staining techniques using flow cytometry. Theriogenology 2003;60:1127-1138.
55. Kavak A, Johannisson A, Lundeheim N, et al: Evaluation of cryopreserved stallion semen from Tori and Estonian breeds using CASA and flow cytometry. Anim Reprod Sci 2003;76:205-216.
56. Kirk ES, Graham JK, Bruemmer JE, Squires EL: Evaluating frozen equine semen by flow cytometry. Anim Reprod Sci 2001;68:348-349.
57. Cross NL, Morales P, Overstreet JW, et al: Two simple methods for detecting acrosome-reacted human sperm. Gamete Res 1986;15:213-226.
58. Cross NL, Meizel S: Methods for evaluating the acrosomal status of mammalian sperm. Biol Reprod 1989;41:635-641.
59. Graham JK, Kunze E, Hammerstedt RH: Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. Biol Reprod 1990;43:55-64.
60. Kavak A, Johannisson A, Malmgren L, et al: Post-thaw evaluation of stallion spermatozoa using triple fluorescent staining and flow cytometry. Anim Reprod Sci 2001;68:349-350.
61. Graham JK: Assessment of sperm quality: a flow cytometric approach. Anim Reprod Sci 2001;68:239-247.
62. Silva PFN: Detection of damage in mammalian sperm cells. Theriogenology 2006;65:958-979.
63. Gravance CG, Garner DL, Miller MG, et al: Fluorescent probes and flow cytometry to assess rat sperm integrity and mitochondrial function. Reprod Toxicol 2001;15:5-10.
64 Cai K, Yang J, Guan M, et al: Single UV excitation of Hoechst 33342 and propidium iodide for viability assessment of rhesus monkey spermatozoa using flow cytometry. Arch Androl 2005;51:371-383.
65. Gravance CG, Garner DL, Baumber J, et al: Assessment of equine sperm mitochondrial function using JC-1. Theriogenology 2000;53:1691-1703.
66. Foster ML, Love CC, Varner DD, et al: Comparison of methods for assessing integrity of equine sperm membranes. Theriogenology 2011;76:334-341.
67. Love CC, Blanchard TL, Varner DD, et al: Effect of daily semen centrifugation and resuspension on the longevity of equine sperm quality following cooled storage. Theriogenology 2012;77:1911-1917.
68. Kiser AM, Sudderth AK, Love CC, et al: Sperm quality following 4 days of cooled storage: impact on fertility [abstract]. J Equine Vet Sci 2012;32:491.
69. Evenson DP, Darzynkiewica Z, Melamed MR: Relation of mammalian sperm chromatin heterogenecity to fertility. Science 1980;210:1131-1133.
70. Love CC: The sperm chromatin structure assay: a review of clinical applications. Anim Reprod Sci 2005;89:39-45.
71. Love CC, Kenney RM: The relationship of increased susceptibility of sperm DNA to denaturation and fertility in the stallion. Theriogenology 1998;57:955-972.
72. Evenson DP, Sailer BL, Jost LK: Relationship between stallion sperm deoxyribonucleic acid (DNA) susceptibility to denaturation in situ and presence of DNA strand breaks: implications for fertility and embryo viability. Biol Reprod Mono 1 1995;6:655-659.
73. Evenson DP, Jost LK, Varner DD: Relationship between sperm nuclear protamine free -SH status and susceptibility to DNA denaturation. J Reprod Fertil Suppl 2000;56:401-406.
74. Love CC, Kenney RM: Scrotal heat stress induces altered sperm chromatin structure associated with decrease in protamine disulfide bonding in the stallion. Biol Reprod 1999;60:615-620.
75. Makhlouf AA, Niederberger C: DNA integrity tests in clinical practice: it is not a simple matter of black and white (or red and green). J Androl 2006;27:316-323.
76. O'Brien J, Zini A: Sperm DNA integrity and male infertility. Urology 2005;65:16-22.
77. Aoki VW, Emery BR, Liu L, et al: Protamine levels vary between individual sperm cells of infertile human males and correlate with viability and DNA integrity. J Androl 2006;27:890-898.
78. Lewis SEM, Aitken RJ: DNA damage to spermatozoa has impacts on fertilization and pregnancy. Cell Tissue Res 2005;322:33-41.
79. Aoki VW, Moskovtsev SI, Willis J, et al: DNA integrity is compromised in protamine-deficient human sperm. J Androl 2005;26:741-748.
80. Sailer BL, Sarkar LJ, Bjordahl JA, et al: Effects of heat stress on mouse testicular cells and sperm chromatin structure. J Androl 1997;18:294-301.
81. Oliva R: Protamines and male infertility. Hum Reprod Update 2006;12:417-435.
82. Fraga CG, Motchnik PA, Shigenaga MK, et al: Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. Proc Natl Acad Sci USA 1991;88:11003-11006.
83. Fatehi AN, Bevers MM, Schoevers E, et al: DNA damage in bovine sperm does not block fertilization and early embryonic development but induces apoptosis after the first cleavages. J Androl 2006;27:176-188.
84. Miller D, Ostermeier GC: Spermatozoal RNA: why is it there and what does it do? Gynecol Obstet Fertil 2006;34:840-846.
85. Miller D, Ostermeier GC: Towards a better understanding of RNA carriage by ejaculate spermatozoa. Hum Reprod Update 2006;12:757-767.

86. Agarwal A, Said TM: Role of sperm chromatin abnormalities and DNA damage in male infertility. Hum Reprod Update 2003;9:331-345.
87. Baumber J, Ball BA, Linfor JJ, et al: Reactive oxygen species and cryopreservation promote deoxyribonucleic acid (DNA) damage in equine sperm. Theriogenology 2003;58:301-302.
88. Chohan KR, Griffin JT, Lafromboise M, et al: Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. J Androl 2006;27:53-59.
89 Erenpreiss J, Spano M, Erenpreisa J, et al: Sperm chromatin structure and male fertility: biological and clinical aspects. Asian J Androl 2006;8:11-29.
90 Evenson DP, Wixon R: Comparison of the Halosperm test kit with the sperm chromatin structure assay (SCSA) infertility test in relation to patient diagnosis and prognosis. Fertil Steril 2005;84:846-849.
91. Linfor JJ, Meyers SA: Detection of DNA damage in response to cooling injury in equine spermatozoa using single-cell gel electrophoresis. J Androl 2002;23:107-113.
92. López-Fernández C, Drespo F, Arroyo F, et al: Dynamics of sperm DNA fragmentation in domestic animals II. The stallion. Theriogenology 2007;68:1240-1250.
93. Teague SR, Bakker AZ, Brehm LM, et al: Comparison of SCSA and TUNEL assays for measurement of stallion sperm DNA quality before and after centrifugation through a silica-particle solution [abstract]. Anim Reprod Sci 2012;121S:205.
94. Serafini R, Varner DD, Bissett W, et al: DNA fragmentation of raw stallion sperm following storage at freezing temperatures [abstract]. J Equine Vet Sci 2012;32:513.
95. Varner DD, Brinsko SP, Blanchard TL, et al: Subfertility in stallions associated with acrosome dysfunction. Proc Annu Conv Am Assoc Equine Pract; 2001. p. 227-228.
96. Brinsko SP, Love CC, Bauer JE, et al: Cholesterol-to-phospholipid ratio in whole sperm and seminal plasma from fertile stallions and stallions with unexplained subfertility. Anim Reprod Sci 2007;99:65-71.
97. Raudsepp T, McCue ME, Das PJ, et al: Genome-wide association study implicates testis-sperm specific FKBP6 as a susceptibility locus for impaired acrosome reaction in stallions. PLos Genet 2012:8;e1003139.
98. Varner DD, Thompson JA, Blanchard TL, et al: Induction of the acrosome reaction in stallion spermatozoa: effects of incubation temperature, incubation time, and ionophore concentration. Theriogenology 2002:58:303-306.
99. Carrell BP: Validation of Pisum sativum agglutinin fluroescent marker for stallion spermatozoa acrosomes with transmission electron microscopy [thesis]. College Station (TX): Texas A&M University 2006. (http://repository.tamu.edu/bitstream/handle/1969.1/ETD-TAMU-2001-THESIS-C365/2001%20Thesis%20C365.pdf?sequence=1).
100. Bosard TS: Flow cytometric evaluation of acrosome function/dysfunction in the stallion[thesis]. College Station (TX): Texas A&M University; 2006. (http://repository.tamu.edu/bitstream/handle/1969.1/ETD-TAMU-1875/BOSARD-THESIS.pdf?sequence=1).
101. Rathi R, Colenbrander B, Bevers MM, et al: Evaluation of in vitro capacitation of stallion spermatozoa Biol Reprod 2001;65:462-470.
102. Varner DD, Ward CR, Storey BA, et al: Induction and characterization of the acrosome reaction in equine spermatozoa. Am J Vet Res 1987;48:1383-1389.
103. Cardullo RA, Florman HM: Strategies and methods for evaluating acrosome reaction. Methods Enzymol 1993:225:136-153.
104. Ward CR, Storey BT: Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. Dev Biol 1984;104:287-296.
105. Brum AM, Thomas AD, Sabeur K, et al: Evaluation of Coomassie blue staining of the acrosome of equine and canine spermatozoa. Am J Vet Res 2006;67:358-362.
106. Cardullo RA, Florman HM: Strategies and methods for evaluating acrosome reaction. Methods Enzymol 1993;225:136-153.
107. Meyers SA, Overstreet JW, Liu IKM, et al: Capaciation in vitro of stallion spermatozoa: comparison of progesterone-induced acrosome reactions in fertile and subfertile males. J Androl 1995;16:47-54.
108. Cheng FP, Gadella BM, Voorhout WF, et al: Progesterone-induced acrosome reaction in stallion spermatozoa is mediated by a plasma membrane progesterone receptor. Biol Reprod 1998;59:733-742.
109. Rathi R, Nielen M, Cheng FP, et al: Exposure of progesterone receptors on the plasma membranes of stallion spermatozoa as a parameter for prediction of fertility. J Reprod Fertil Suppl 2000;56:87-91.
110. Ball BA, Fagnan MS, Dobrinski I: Determination of acrosin amidase activity in equine spermatozoa. Theriogenology 1997;48:1191-1198.
111. Kennedy WP, Kaminski JM, Van der Ven HH, et al: A simple, clinical assay to evaluate the acrosin activity of human spermatozoa. J Androl 1989;10:221-231.
112. Cross NL: A modified and improved assay for sperm amidase activity. J Androl 1990;11:409413.
113. Gamboa S, Ramalho-Santos J: SNARE proteins and caveolin-1 in stallion spermatozoa: possible implications for fertility. Theriogenology 2005;64:275-291.
114. Sousa APM, Gomes-Santos CSS, Ramalho-Santos J: Localization of snares, NSF and Caveolin 1 in human spermatozoa: relationship with seminal parameters. Arch Androl 2006;52:347-353.

115. Desvousges AL, Dow CA, Hayna JT, et al: Heat shock induces apoptosis in equine spermatozoa. Anim Reprod Sci 2006;94:125-126.
116. Brum A, Sabeur K, Ball BA: Apoptotic-like changes in equine spermatozoa after separation by density-gradient centrifugation or after cryopreservation. Anim Reprod Sci 2006;94:138-139.
117. Wang X, Sharma RK, Sikka SC, et al: Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. Fertil Steril 2003;80:531-535.
118. Said TM, Paasch U, Glander H-J, et al: Role of caspases in male infertility. Hum Reprod Update 2004;10:39-51.
119. Topfer-Petersen E, Ekhasi-Hundrieser M, Kirchhoff C, et al: The role of stallion seminal proteins in fertilisation. Anim Reprod Sci 2005;89:159-170.
120. Bellin ME, Oyarzo JN, Hawkins HE, et al: Fertility-associated antigen on bull sperm indicates fertility potential. J Anim Sci 1998;76:2032-2039.
121. Buffone MG, Calamera JC, Verstraeten SV, et al: Capacitation-associated protein tyrosine phosphorylation and membrane fluidity changes are impaired in the spermatozoa of asthenozoospermic patients. Reproduction 2005;129:697-705.
122. Yunes R, Doncel GF, Acosta AA: Incidence of sperm-tail tyrosine phophorylation and hyperactivated motility in normozoospermic and asthenozoospermic human sperm samples. Biocell 2003;27:29-36.
123. Tardif S, Dube C, Chavalier S, et al: Capacitation is associated with tyrosine phosphorylation and tyrosine kinase-like activity in pig sperm proteins. Biol Reprod 2001;65:784-792.
124. Burnaugh L, Sabeur K, Ball BA: Generation of superoxide anion by equine spermatozoa as detected by dihydroethidium. Theriogenology 2007;67:580-589.
125. Bianchi PG, Manicardi GC, Urner F, et al: Chromatin packaging and morphology in ejaculated human sprmatozoa: evidence of hidden anomalies in normal spermatozoa. Mol Hum Reprod 1996;2:139-144.
126. Nasr-Esfahani MH, Razavi S, Mardani M: Relation between different human sperm nuclear maturity tests and in vitro fertilization. J Assist Reprod Genet 2001;18:219-225.
127. Iranpour FG, Nasr-Esfahani MH, Yalojerdi MR, et al: Chromomycin A_3 staining as a useful tool for evaluation of male fertility. J Assist Reprod Genet 2000;17:60-66.
128. Franken DR, Franken CJ, de la Guerre H, et al: Normal sperm morphology and chromatin packaging: comparison between aniline blue and chromomycin A3 staining. Andrologia 1999;31:361-366.
129. Gradil C, Yoon S-Y, Brown J, et al: PLC-Zeta: A marker of fertility for stallions. Anim Reprod Sci 2006;94:23-25.
130. Swann K, Saunders CM, Rogers NT, et al: PLCzeta: a sperm protein that triggers calcium ion oscillations and egg activation in mammals. Semin Cell Dev Biol 2006;17:264-273.
131. Bedford-Guaus SJ, McPartlin LA, Varner DD: Characterization of equine phospholipase C zeta: a review and preliminary results on expression defects in subfertile stallions. J Equine Vet Sci 2012;32:445-450.
132. Neild DM, Gadella BM, Colenbrander B, et al: Lipid peroxidation in stallion spermatozoa. Theriogenology 2002;58:295-298.
133. Almeida J, Ball BA: Effect of alpha-tocopherol and tocopherol succinate on lipid peroxidation in equine spermatozoa. Anim Reprod Sci 2005;87:321-337.
134. Neild DM, Brouwers JFHM, Colenbrander B, et al: Lipid peroxide formation in relation to membrane stability of fresh and frozen thawed stallion spermatozoa. Mol Reprod Dev 2005;72:230-238.
135. Guthrie HD, Welch GR: Use of fluorescence-activated flow cytometry to determine membrane lipid peroxidation during hypothermic liquid storage and freeze-thawing of viable boar sperm loaded with C11-BODIPY 581/591. J Anim Sci 2007;85:1402-1411.
136. Brouwers JFHM, Gadella BM: In situ detection and localization of lipid peroxidation in individual bovine sperm cells. Free Radic Biol Med 2003;35:1382-1391.
137. Brouwers JF, Silva PFN, Gadella BM: New assays for detection and localization of endogenous lipid peroxidation products in living boar sperm after BTS dilution or after freeze-thawing. Theriogenology 2005;63:458-469.
138. Roca J, Gil MA, Hernandez M, et al: Survival and fertility of boar spermatozoa after freeze-thawing in extender supplemented with butylated hydroxytoluene. J Androl 2004;25:397-405.
139. Stradaioli G, Gennevieve A, Magistrini M: Evaluation of lipid peroxidation by thiobarbituric acid test in equine spermatozoa. Anim Reprod Sci 2001;68:320-322.
140. Stradaioli G, Magistrini M: A comparative evaluation of thiobarbituric acid methods for determination of malondialdehyde in equine spermatozoa. Theriogenology 2002;58:347-350.
141. Aitken RJ, Harkiss D, Buckingham DW: Analysis of lipid peroxidation mechanisms in human spermatozoa. Mol Reprod Dev 1993;35:302-315.
142. Martinez P, Proverbio F, Camejo MI: Sperm lipid peroxidation and pro-inflammatory cytokines. Asian J Androl 2007;9:102-107.
143. Agarwal A, Prabakaran SA: Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. Indian J Exp Biol 2005;43:963-974.
144. Peris SI, Bilodeau JF, Dufour M, et al: Impact of cryopreservation and reactive oxygen species on DNA integrity, lipid peroxidation, and functional parameters in ram sperm. Mol Reprod Dev 2007;74:878-892.
145. Kumar TR, Muralidhara: Induction of oxidative stress by organic hydroperoxides in testis and epididymal sperm of rats in vivo. J Androl 2007;28:77-85.

146. Stradaioli G, Rubei M, Zamparini M, et al: Enzymatic evaluation of phospholipid hydroperoxide glutathione peroxidase (PHGPx) in equine spermatozoa. Anim Reprod Sci 2006;94:29-31.
147. Foresta C, Flohe L, Garolla A, et al: Male fertility is linked to the selenoprotein phospholipid hydroperoxide glutathione peroxidase. Biol Reprod 2002;67:967-971.
148. Stradaioli G, Parillo F, Sylla L, et al: Enzymatic and immunohistochemical evaluation of phospholipid hydroperoxide glutathione peroxidase (PHGPx) in bovine spermatozoa. Proc Int Cong Anim Reprod 2004;I:192.
149. Weir CP: Spermatozoa have decreased antioxidant enzymatic capacity and increased reactive oxygen species production during aging in the brown Norway rat. J Androl 2007;28:229-240.
150. Mesequer M, de los Santos MJ, Simon C, et al: Effect of sperm glutathione peroxidases 1 and 4 on embryo asymmetry and blastocyst quality in ooctye donation cycles. Fertil Steril 2006;86:1376-1385.
151. Aitken RJ, Clarkson JS, Hargreave TB, et al: Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligozoospermia. J Androl 1989;10:214-220.
152. Aitken RJ, Clarkson JS, Fishel S: Generation of reactive oxygen species lipid peroxidation and human sperm function. Biol Reprod 1989;40:183-197.
153. Ball BA, Vo AT, Baumber J: Generation of reactive oxygen species by equine spermatozoa. Am J Vet Res 2001;62:508-515.
154. Ball BA, Baumber J, Vo A, et al: Effect of oxidative stress on equine spermatozoa. Anim Reprod Sci 2001;68:364.
155. Guthrie HD, Welch GR: Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. J Anim Sci 2006;84:2089-2100.
156. Said TM, Agarwal A, Sharma RK, et al: Human sperm superoxide anion generation and correlation with semen quality in patients with male infertility. Fertil Steril 2004;82:871-877.
157. Aitken RJ, Irvine DS, Wu FC: Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. Am J Obstet Gynecol 1991;164:542-551.
158. Whittington K, Harrison SC, Williams KM, et al: Reactive oxygen species (ROS) production and the outcome of diagnostic tests of sperm function. Int J Androl 1999;22:236-242.
159. Pesch S, Bergmann M, Bostedt H: Determination of some enzymes and macro- and microelements in stallion seminal plasma and their correlations to semen quality. Theriogenology 2006;66:307-313.
160. Mollova M, Atanassov B, Nedkova R, et al: Biochemical and immunochemical characterization of boar sperm flagellar protein with role in hyperactivation/capacitation process. Reprod Biol 2006;6:79-94.
161. Yousef MI, Esmail AM, Baghdadi HH: Effect of isoflavones on reproductive performance, testosterone levels, lipid peroxidation, and seminal plasma biochemistry of male rabbits. J Environ Sci Health B 2004;39:819-833.
162. Noquera Velasco JA, Tovar Zapata I, Martinez Hernandez P, et al: Lactic dehydrogenase-C4 activity in seminal plasma and male infertility. Fertil Steril 1993;60:331-335.
163. Huszar G, Corrales M, Vigue L: Correlation between sperm creatine phosphokinase activity and sperm concentration in normospermic and oligospermic men. Gamete Res 1988;19:67-75.
164. Huszar G, Vigue L: Incomplete development of human spermatozoa is associated with increased creatine phosphokinase concentration and abnormal head morphology. Mol Reprod Dev 1993;34:292-298.
165. Huszar G, Stone K, Dix D, et al: Putative creatine kinase M-isoform in human sperm is identified as the 70-kilodalton heat shock protein HspA2. Biol Reprod 2000;63:925-932.
166. Huszar G, Ozkavukcu S, Jakab A, et al: Hyaluronic acid binding ability of human sperm reflects cellular maturity and fertilizing potential: selection of sperm for intracytoplasmic sperm injection. Curr Opin Obstet Gynecol 2006;18:260-267.
167. Cuasnicu PS, Ellerman DA, Cohen DJ, et al: Molecular mechanisms involved in mammalian gamete fusion. Arch Med Res 2001;32:614-618.
168. Ellerman DA, Cohen DJ, Da Ros VG, et al: Sperm protein "DE" mediates gamete fusion through an evolutionarily conserved site of the CRISP family. Dev Biol 2006;297:228-237.
169. Reineke A, Hess O, Schambony A, et al: Sperm-associated seminal plasma proteins - a novel approach for the evaluation of sperm fertilizing ability of stallions. Pferdcheikunde 1999;6:531-537.
170. Roberts KP, Ensrud KM, Wooters JL, et al: Epididymal secreted protein Crisp-1 and sperm function. Mol Cell Endocrinol 2006;250:122-127.
171 Schambony A, Hess O, Gentzel M, et al: Expression of CRISP proteins in the male equine genital tract. J Reprod Fertil Suppl 1998;53:67-72.
172. Jude R, Giese A, Piumi F, et al: Molecular characterization of the CRISP 1 gene-a candidate gene for stallion fertility. Theriogenology 2002;58:417-420.
173. Meyers SA, Rosenberger AE: A plasma membrane-associated hyaluronidase is localized to the posterior acrosomal region of stallion sperm and is associated with spermatozoal function. Biol Reprod 1999;61:444-451.
174. Meyers SA, Rosenberger A, Orpneck K: Localization and cellular distribution of a unique hyaluronidase in stallion spermatozoa during epididymidal transit. J Reprod Fertil Suppl 2000;56:79-86.
175. Meyers SA: Equine sperm-oocyte interaction: the role of sperm surface hyaluronidase. Anim Reprod Sci 2001;68:291-303.

176. Jonakova V, Manaskova P, Kraus M, et al: Sperm surface proteins in mammalian fertilization. Mol Reprod Dev 2000;56 (Suppl. 2):277.
177. Rodriguez-Martinez H, Iborra A, Martinez P, et al: Immunoelectronmicroscopic imaging of spermadhesin AWN epitopes on boar spermatozoa bound in vivo to the zona pellucida. Reprod Fertil Dev 1998;10:491-497.
178. Jonakova V, Kraus M, Veselsky L, et al: Spermadhesins of the AQN and AWN families, DQH sperm surface protein and HNK protein in the heparin-binding fraction of boar seminal plasma. J Reprod Fertil 1998;114:25-34.
179. Boue F, Sullivan R: Cases of human infertility are associated with the absence of P34H an epididymal sperm antigen. Biol Reprod 1996;54:1018-1024.
180. Sullivan R, Legare C, Villeneuve M, et al: Levels of P34H, a sperm protein of epididymal origin, as a predictor of conventional in vitro fertilization outcome. Fertil Steril 2006;85:1557-1559.
181. Xia WY, Huang YF, Xu XF: Epididymal sperm protein P34H and male reproduction. Zhonghua Nan Ke Xue 2002;8:356-358.
182. Sullivan R: Male fertility markers, myth or reality. Anim Reprod Sci 2004;82-83:341-347.
183. Bailey LB, Brady HB, Tardif S, et al: Zonadhesin expression and localization in stallion testes. Anim Reprod Sci 2006;94:56-59.
184. Breazeale KR, Brady HA, Bi M, et al: Biochemical properties and localization of zonadhesin in equine spermatozoa. Theriogenology 2002;58:359-362.
185. Hardy DM, Garbers DL: A sperm membrane protein that binds in a species-specific manner to the egg extracellular matrix is homologous to von Willebrand factor. J Biol Chem 1995;270:26025-26028.
186. Bi M, Hickox JR, Winfrey VP, et al: Processing, localization and binding activity of zonadhesin suggest a function in sperm adhesion to the zona pelucida during exocytosis. Biochem J 2003;375:477-488.
187. Lea IA, Sivashanmuqam P, O'Rand MG: Zonadhesin: characterization, localization, and zona pellucida binding. Biol Reprod 2001;65:1691-1700.
188 Gao A, Garbers DL: Species diversity in the structure of zonadhesin, a sperm-specific membrane protein containing multiple cell adhesion molecule-like domains. J Biol Chem 1998;273:3415-3421.
189 Eager FE, Brady HA, Thompson LD, et al: Biochemical properties of zonadhesin in spermatozoa of fertile and subfertile stallions. Proc Equine Sci Soc 2005;19:95-100.
190. Tardif S, Brady HA, Breazeale KR, et al: Zonadhesin D3-polypeptides vary among species but are similar in Equus species capable of interbreeding. Biol Reprod 2010;82:413-421.
191. Klinefelter GR, Welch JE, Perreault SD, et al: Localization of the sperm protein SP22 and inhibition of fertility in vivo and in vitro. J Androl 2002;23:48-63.
192. Miller LMJ, Greene ES, Roberts KP, et al: Expression of equine sperm protein 22kDa (SP22) in testicular and epididymal tissue. Anim Reprod Sci 2006;94:54-55.
193. Miller LT, Troedsson MH, Duoos LA, et al: Immunocytochemical detection and localization of sperm protein 22 (SP22) in fresh and cryopreserved equine semen. Biol Reprod 2003;68 (Suppl.1):166-167.
194. Wrench N, Pinto CRF, Klinefelter GR, et al: Seasonal expression and pattern of SP22 immunolocalization in stallion semen. Anim Reprod Sci 2006;94:32-35
195. Mann T, Lutwak-Mann C. Male reproductive function and semen. Themes and trends in physiology, biochemistry and investigative andrology. New York: Springer-Verlag; 1981.
196. Juyena NS, Stelletta C. Seminal plasma: an essential attribute to spermatozoa. J Androl 2012:33:536-551.
197. Caballero I, Parrilla I, Almiñana C, et al: Seminal plasma proteins as modulators of the sperm function and their application in sperm biotechnologies. Reprod Domest Anim 2012;47 Suppl 3:12-21.
198. Varner DD, Blanchard TL, Love CL, et al: Effects of semen fractionation and dilution ratio on equine spermatozoal motility parameters. Theriogenology 1987;28:709-723.
199. Jasko DJ, Moran DM, Farlin ME, et al: Effect of seminal plasma dilution or removal on spermatozoal motion characteristics of cooled stallion semen. Theriogenology 1991;35:1059-1067.
200. Rigby SL, Brinsko SP, Cochran M, et al: Advances in cooled semen technologies: seminal plasma and semen extender. Anim Reprod Sci 2001;68:171-180.
201. Love CC, Brinsko SP, Rigby SL, et al: Relationship of seminal plasma level and extender type to sperm motility and DNA integrity. Theriogenology 2005;63:1584-1591.
202. Love CC, Thompson JA, Brinsko SP, et al: Effect of inter-stallion seminal plasma variability on motility, viability, and DNA integrity of cauda epididymal sperm [abstract]. Anim Reprod Sci 2010;121S:191.
203. Varner DD, Love CC, Blanchard TL, et al: Breeding-management strategies and semen-handling techniques for stallions: case scenarios. Proc Annu Conv Am Assoc Equine Pract; 2010. p. 215-226.
204. Padilla AW, Foote RH: Extender and centrifugation effects on the motility patterns of slow-cooled stallion spermatozoa. J Anim Sci 1991;69:3308-3313.

Influence of modified 2-point temperament score on AI pregnancy in beef heifers

Stephanie Schroeder, Ram Kasimanickam
Department of Veterinary Clinical Science, College of Veterinary Medicine, Washington State University, Pullman, WA

Temperament is described as the reacting characteristics of cattle when exposed to human handling. Excitable temperament has been alleged to have detrimental effects on both production and reproduction parameters. The objective of this study was to evaluate the effect of temperament, determined by chute exit and gait, on artificial insemination (AI) pregnancy rates in beef heifers following fixed time A.I. The hypothesis was excitable temperament will lower AI pregnancy rates. Angus cross beef heifers (N=947) at eight locations were the subjects in this study. At the initiation of synchronization (Day 0), all heifers received a body condition score (BCS; 1=emaciated; 9=obese), and temperament score (0=calm; slow exit and walk); (1= excitable; fast exit or jump or trot or run). Heifers were synchronized with 5-day CO-Synch+ controlled internal drug release (CIDR) protocol. Blood samples were collected at both the instigation of synchronization and the time of AI for cortisol measurement. Briefly, all heifers received a CIDR (Eazi-Breed™ CIDR® Cattle Insert; Pfizer Animal Health, New York, NY) on Day 0. At each farm, heifers were randomly divided into two groups: one group received 100μg of gonadorelin hydrochloride (GnRH; Factrel®, Pfizer Animal Health), while the other group received no treatment at the time of CIDR insertion at Day 0. On Day 5, CIDR inserts were removed and all heifers received 25 mg of prostaglandin $F_{2\alpha}$ (PGF; Lutalyse® sterile solution; Pfizer Animal Health). The GnRH and no treatment groups were further divided into 1PGF and 2PGF groups. The heifers in the 2PGF group received a second dose of PGF six hours after the initial injection. All heifers were inseminated 56 hours after CIDR removal, and received 100μg of GnRH at this time. Two weeks later, intact Angus bulls were placed with the heifers (approximately 1:40 to 1:50 bull:cow ratio) across all treatment groups. The bulls were left with the heifers for the remainder of the 60 to 70 day breeding season. Heifers were examined for pregnancy via ultrasound 70 days after AI to determine the time of conception. The data were analyzed using PROC MIXED procedure of SAS (SAS Version 9.3, Cary, NC). The variables included were as follows: synchronization treatments, exit score (excitable versus calm), locations (1 to 8), BCS (≤6 vs. >6), and 2-way interactions. Artificial insemination sires and AI technicians were offered as random effect in the model. The *P* value was set at > 0.1 for exclusion and $\alpha \leq 0.05$ for significance.

The proportion of heifers determined to have excitable temperaments varied across locations (27.9 to 78.9%; P<0.01). Accounting for locations (P=0.06), synchronization treatment (P=0.03), and location by exit score interaction (P<0.01), the heifers with excitable temperament had lower AI pregnancy compared to heifers with calm temperament (51.9 [331] vs. 60.3% [636]; P=0.006). The AI pregnancy rates ranged from 50.3 to 58.3% for synchronization treatments. The AI pregnancy for locations varied from 50 to 62.4%. Blood serum cortisol concentration between calm and excitable heifers significantly differed at the initiation of synchronization (4.43 and 5.43 ng/mL) and did not differ at AI (4.11 and 4.2 ng/mL). Inter- and intra rater agreement for exit scoring were moderate and good (Kappa=0.596 and 0.797, respectively). The predictive value for calm and pregnant was 0.87, and excited and pregnant was 0.76.

In conclusion, heifers with excitable temperament lowered AI pregnancy rates in the beef operation. The modified 2-point temperament scoring method can be used to accurately identify heifers with excitable temperament.

Keywords: Beef heifers, temperament, exit score; AI pregnancy

Cesarean section in alpacas and llamas at a referral center - technique, survival, and postoperative fertility: 24 cases (2000-2012)

A.J. Campbell, L.K. Pearson, A. Tibary
Comparative Theriogenology, Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University Pullman, WA

Cesarean section may be indicated during late gestation in alpacas and llamas to resolve dystocia or remove a non-viable fetus. The objective of this retrospective study was to evaluate indications for cesarean section in alpacas and llamas, surgical techniques employed, dam and cria survival rates, and postoperative fertility. Additional data included time to presentation, anesthetic protocol used, parity, and gestational age.

Twenty-one alpacas and three llamas met the inclusion criteria for the study. Mean (± SD) age of females was 5.1 ± 2.3 years (range 2-10). There was not a significant difference between maiden females (45.8%, n=11) and multiparous females (54.2%, n=13) ($p>0.05$). The mean gestational age at the time of presentation was 353.8 ± 11.8 days (range 329-376). The time to referral ranged from 0-72 hours. Cesarean section was performed due to dystocia (95.8%; n=23) or concurrent maternal disease (4.2%; n=1). Uterine torsion (60.9%; n=14) was identified as the predominant cause of dystocia. Other causes of dystocia identified were bilateral hip flexion (21.7%; n=5), posterior presentation (4.3%; n=1), failure of cervical dilation (8.7%, n=2), and the presence of twins (4.3%; n=1).

Cesarean section was performed via ventral midline approach (79.2%; n=19) or left paralumbar fossa approach (20.8%; n=5); two of the cases utilizing a paralumbar fossa approach were llamas. The most common anesthetic protocols used butorphanol, diazepam, and propofol. All cases were intubated and maintained on isoflurane or sevoflurane in oxygen.

Overall survival rate was 91.2% (n=22) for females and 45.8% (n=11) for crias. One female alpaca died during recovery from anesthesia and one was euthanized postoperatively.

The most frequent postoperative complication was retained placenta (8.3%; n=2). Other complications included one case each (4.2%) of radial nerve paralysis, sepsis, and anemia. Radial nerve paralysis and sepsis occurred in the same case. All postoperative complications were resolved prior to discharge from the hospital. Of the surviving females (n=22), reproductive records were available for 11 alpacas and two llamas following cesarean section (50%). Ten females were bred at least once following cesarean section, and seven of those (70%) delivered at least one cria. No breedings were attempted in three females following cesarean section.

In the authors' referral center, dystocia due to uterine torsion or bilateral hip flexure is the most common indication for cesarean section in alpacas and llamas. Survival of the dam is excellent. Postoperative fertility following cesarean section is generally good but more observations are needed to determine risk factors for loss of fertility and cria survival.

Keywords: Camelid; uterine torsion; dystocia; retained placenta; surgery

Equine lactoferrin increases *in vitro* binding of polymorphonuclear neutrophils to spermatozoa

A. Esteller-Vico, K.E. Scoggin, E.L. Squires, B.A. Ball, M.H.T. Troedsson
Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY

Following insemination, spermatozoa induce a migration of polymorphonuclear neutrophils (PMNs) to the uterine lumen, which play a major role in resolution of the post-mating inflammatory response. Equine lactoferrin is an iron binding glycoprotein (~ 80 kDa) that is present in large amounts in the seminal plasma of the stallion and in the endometrium of the mare during estrus. Lactoferrin plays an important role in modulating immune and inflammatory responses, particularly as a mediator of cell-to-cell interactions. Due to its role in host defense mechanisms and its high concentration in seminal plasma, we hypothesized that lactoferrin modulates PMN-sperm interactions in the uterus. The objectives of this study were: 1) to characterize the role of lactoferrin in the post-mating inflammatory response of the uterus and 2) to characterize the expression of lactoferrin mRNA and protein in the testis and epididymis of stallions.

Methods

An *in vitro* binding assay between PMNs and sperm cells was performed in triplicate and was used to evaluate the immune response. PMNs were isolated from healthy mares and incubated for 30 min with dead sperm stained with propidium iodide (PI) in the presence of medium (Hank's balanced salt solution), seminal plasma (pooled from four stallions) or purified lactoferrin. Samples were analyzed using flow cytometry, gating on PMNs by forward and side scatter (FSC-SSC) and on sperm based on the PI fluorescence signal (FL-2). Lactoferrin mRNA expression was characterized by qPCR and protein expression was characterized by immunohistochemistry and western blots from the testes and epididymides (caput, corpus and cauda) of four stallions (≥ 2-years-old). Data were tested for normality and repeated measures ANOVA was used for comparison of treatment effects.

Results

Both lactoferrin ($62.5 \pm 3.3\%^{a}$) and seminal plasma ($76.5 \pm 0.3\%^{a}$) treatments significantly ($P<0.05$) enhanced binding of PMNs to dead sperm after 30 min of incubation as compared to control medium treatment ($42.9 \pm 1.6\%^{b}$). Lactoferrin mRNA was highly expressed in the corpus epididymis, followed by the cauda and caput epididymis, but was absent from the testis. These results were confirmed by immunohistochemistry with prominent immunoexpression in the luminal epithelium of the corpus epididymis, followed by cauda, caput epididymis, with no expression in the testis. Lactoferrin protein was present in tissue extracts from the cauda and corpus epididymis, but not from the caput epididymis or testis.

Conclusions

Lactoferrin is a seminal plasma protein that is primarily expressed and produced in the corpus and cauda of the epididymis of stallions. Seminal plasma enhances binding of PMNs to dead sperm cells *in vitro* and purified lactoferrin alone was able to reproduce this effect. These findings suggest that one of the roles of lactoferrin in the reproductive tract of the mare is to enhance cell-to-cell interactions between PMNs and nonviable sperm, which *in vivo,* might lead to the resolution of post-breeding endometritis.

Keywords: Lactoferrin, post-breeding endometritis, neutrophils, spermatozoa, equine

Os clitoridis presence on radiographs submitted for coxofemoral dysplasia evaluations

Michelle Kutzler,[a] G. Gregory Keller,[b] Fran Smith[b,c]
[a]Department of Animal Science, Oregon State University, Corvallis, OR; [b]Orthopedic Foundation for Animals, Columbia, MO; [c]Smith Veterinary Hospital, Burnsville, MN

Introduction

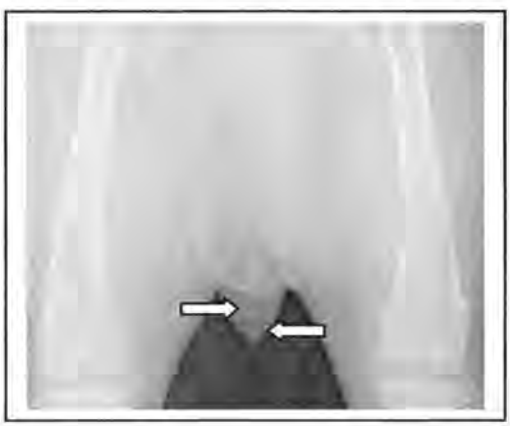
The os clitoridis can occasionally be found radiographically in bitches.[1] However, some authors have associated the presence of an os clitoridis in bitches with an intersex or masculinized condition.[1,2] Therefore, the objective of this study was to determine the incidence of an os clitoridis in bitches. The hypothesis was that an os clitoridis is present in normal bitches, albeit at a low incidence.

Methods

Ventrodorsal radiographs submitted to the Orthopedic Foundation for Animals for coxofemoral dysplasia evaluations and determined to have "normal" hips were used for this study. Because of the potential difficulty in confirming the presence (or absence) of an os clitoridis in dogs with tails obscuring the perineal area on the ventrodorsal view, only dogs with docked tails were used (American Cocker Spaniels (n=200) and German Short-hair Pointers (n=200)). If an os clitoridis was visualized radiographically, its shape and degree of radio-opacity were recorded.

Results

In 3% (6/200) American Cocker Spaniels and 2% (4/200) German Shorthair Pointers, the presence of an os clitoridis in the tip of the vulva could be confirmed on a single ventrodorsal radiograph. In one bitch, two mineralized opacities were present within the vulva, one at the tip and another slightly more cranial (arrows on figure below). The shape of the os clitoridis ranged from circular to oblong and the degree of opacity was always slightly less than that of the cortical coccygeal bone.



Discussion

This study has shown that an os clitoridis can be present in bitches assumed to be reproductively normal. An os clitoridis has been reported in other mammals, including fossa (*Cryptoprocta ferox*),[3] ring-tailed lemur (*Lemur catta*)[4] and some strains of mice. An os clitoridis has also been reported in members of all three pinniped families, but its appearance, even within a single species, is irregular.[5]

References

1. Wucherer K, Lulich JP: Os clitoris or os penis with ambiguous genitalia. J Am Vet Med Assoc 2006; 229:1383-1384.
2. Grandage J, Robertson BF: An os clitoridis in a bitch. Aust Vet J 1971; 47:346-347.
3. Hawkins CE, Dallas JF, Fowler PA, et al: Transient masculinization in the fossa, *Cryptoprocta ferox* (Carnivora, Viverridae). Biol Reprod 2002; 66:610-615.
4. Drea CM, Weil A: External genital morphology of the ring-tailed lemur (*Lemur catta*): females are naturally "masculinized". J Morphol 2008; 269:451-463.
5. King JE: Seals of the world. Ithaca: Cornell University Press; 1983. p. 182-183.

Stallion sperm recovery rate after centrifugation and removal of the supernatant using different methods

V.C.L. Gomes,[a] L.M.J. Miller,[b] J. Bradford,[b] S. Holliday,[c] S. Skinner,[c] M.S. Ferrer[b]
[a]CAPES Foundation, Ministry of Education of Brazil, Brasilia, DF; [b]Department of Clinical Sciences, College of Veterinary Medicine and [c]Developing Scholars Program, Kansas State University, Manhattan, KS

A reduction in sperm loss during centrifugation would yield an increase in viable sperm for cryopreservation or insemination. It was hypothesized that the method used to remove the supernatant after centrifugation would have an effect on sperm recovery rate. Some methods may reduce processing time and, therefore, sperm loss in the supernatant due to swim up of viable sperm from the pellet. Two experiments were conducted to test this hypothesis. In each experiment, two ejaculates were collected from each of three stallions, totaling six ejaculates per experiment. Semen was extended to 25×10^6 sperm/mL with a milk based semen extender, divided into three 40 mL aliquots, and centrifuged for 10 min at 900 × g in 50 mL conical centrifugation tubes. Then, 37 mL of supernatant was removed using one of three methods. In Exp. 1, the supernatant was removed with a 1.5 mL glass Pasteur pipette attached to a latex bulb (GPP), a 3 mL plastic transfer pipette (PTP), or vacuum suction (VS). Since the GPP yielded the best recovery rate in the first experiment, this method was subsequently compared with others in the second experiment. In Exp. 2, the supernatant was removed with the GPP, a 10 mL pipette attached to a manual pipettor (10P), or a combination of the 10P up to the 10 mL mark of the centrifugation tube and GPP thereafter (10PGPP). Concentration was determined with a hemacytometer in both the pre-centrifuged samples and the removed supernatants, and the sperm recovery rate was calculated. The means were compared among groups using ANOVA for repeated measurements. In Exp. 1, mean sperm recovery rate was higher in the GPP (83.58 ± 10.61%) than in PTP (66.61 ± 11.68%) or VS (61.62 ± 15.79%) groups ($P < 0.05$) (Mean ± SD). In Exp. 2, there were no differences in sperm recovery rates among groups GPP (92.15 ± 9.78%), 10P (91.81 ± 11.20%), and 10PGPP (75.62 ± 25.04%) ($P > 0.05$). Thus, the method used to remove the supernatant had an effect on sperm recovery rate, and the 1.5 mL glass Pasteur pipette yielded one of the highest rates, even with a longer processing time and the need of repeated pipetting. Therefore, the difference in sperm recovery rate may be due to changes in negative pressure or rate of fluid flow with the various methods rather than by processing time and potential for swim up. In conclusion, the glass Pasteur pipette, the 10 mL pipette, and the combination of both provided the highest sperm recovery rates after centrifugation and removal of the supernatant. The high recovery rate associated with the practicality in removing the supernatant with a 10 mL pipette makes this method recommended.

Keywords: Equine, centrifugation, pipette, supernatant removal, sperm recovery

The effect of immune modulators on endometrial cytokine expression in mares susceptible to persistent breeding induced endometritis

E.M. Woodward,[a] E.S. Metcalf,[b] K.E. Scoggin,[a] M. Christoffersen,[c] D.W. Horohov,[a] E.L. Squires,[a] P.A. Clausen,[d] M.H.T. Troedsson[a]

[a]The Maxwell H. Gluck Equine Research Center, University of Kentucky, Lexington, KY; [b]Honahlee PC, Sherwood, OR; [c]Dept. of Large Animal Sciences, Health and Medical Sciences, University of Copenhagen, DK; [d]Cytomedix Inc., Gaithersburg, MD

Persistent breeding induced endometritis (PBIE), is a leading reproductive health concern in horses. Treatment strategies aimed at modulating the innate immune response are available, but the mechanisms of action are not well described. The objective of this study was to evaluate the effects of treatment with three immunomodulators: (1) dexamethasone, (2) mycobacterial cell wall extract (MCWE) and (3) platelet rich plasma (PRP) on the endometrial innate immune response in mares susceptible to PBIE. We hypothesized that these treatments alter uterine NO production and the mRNA expression of IL1B, IL6, IL10, IFNG, IL1RN, and iNOS when compared to non-treated susceptible mares. In the first experiment, artificial insemination (AI) was performed in six mares susceptible to PBIE during three consecutive estrous cycles with 1 $\times$ 10^9 killed spermatozoa 1) alone (control), or in combination with 2) dexamethasone (50 mg iv) at the time of AI, or 3) with MCWE (Settle™, 1.5 mg iv) administered 24 hours prior to AI. Uterine secretions were collected six hours after AI using a sterile tampon, and a 200 mL lavage was used to collect remaining secretions. An endometrial biopsy was obtained immediately after uterine fluid collection and stored in RNAlater® until further processing. NO concentrations in the uterine secretions were measured using a commercial NO assay, and total intrauterine NO was calculated using the $C_1V_1 = C_2V_2$ equation. Inflammatory cytokines in uterine biopsies were determined by qPCR. Data were log10 transformed and analyzed with an ANOVA. Total intrauterine NO was decreased after treatment with MCWE ($P = 0.047$), but dexamethasone had no effect on intrauterine NO. Expression of IL1B mRNA was lower after treatment with dexamethasone ($P < 0.001$) and MCWE ($P = 0.046$) when compared to control. IFNG mRNA expression tended to decrease after treatment with dexamethasone ($P = 0.079$), but no differences were detected in the mRNA expression of other cytokines after any of the treatments. A second experiment evaluated the effect of PRP on the endometrial response to AI in the clinical setting. Nine mares with a history of PBIE were bred over two consecutive cycles with >2 x 10^8 progressively motile spermatozoa 1) alone or 2) with PRP (2-3 mL of PRP brought to a final volume of 10 mL with platelet poor plasma) administered iu 24 hours prior to AI. Endometrial biopsies were collected 24-36 hours after AI, and stored in RNAlater®, before processed for qPCR. Data were analyzed using Wilcoxon signed-rank tests. mRNA expression of IL1B, IL6, and iNOS were decreased from non treated cycles ($P \leq 0.02$), and expression of IL8 tended to decrease after treatment ($P = 0.06$). No differences were detected for IL1RN, TNFA, or IL10. In conclusion, although immune modulators may act through different mechanisms, they appear to aid mares in restoring a balance of pro- and anti-inflammatory mediators in mares with PBIE.

Keywords: Endometritis, cytokines, dexamethasone, MCWE

A placental inflammatory reaction to LPS at 34 days or near-term is inhibited by the non-steroidal anti-inflammatory drug flunixin meglumine

C.S. Bailey, W. Brown, J. Vasgaard, K. McGinnis, M.K. Diaw, C.A. Bare, M. Lewitt, J. Gadsby
College of Veterinary Medicine, North Carolina State University, Raleigh NC

Infectious and inflammatory conditions are among the most common causes of pregnancy loss in the horse and have been the focus of a wide group of researchers for several decades. Through this work, the endometrial and luteal responses to infection have been well-documented, as well as the ultimate pregnancy-outcome after exposure to either bacterial or inflammatory mediators during early and late gestation. However, the role of the conceptus as an active participant in the pathogenesis of disease has not been elucidated.

We hypothesized that both embryonic and term placenta would respond to *Escherichia coli*-derived lipopolysaccharide (LPS) through production of prostaglandin (PG) in a controlled, *ex vivo* environment. We further hypothesized that the nonsteroidal anti-inflammatory drug flunixin meglumine (FM) would suppress LPS-induced prostaglandin production.

Six embryos were collected at 34 days of gestation as previously described.[1] In addition, placentae from two near-term mares were collected surgically between 300 and 330 days. Placental tissues were incubated in triplicate for each mare for 24 hours in control medium, 1 μg/mL LPS, 10 μmol/L FM or 1 μg/mL LPS + 10 μmol/L FM. Medium samples from each well were processed for PGE and PGF2α using commercial EIA-kits (Cayman Chemical, Ann Arbor, MI).

Treatment with LPS resulted in a significant increase in PGF2α production in embryonic tissues, while FM significantly reduced PGF2α production ($p=0.00001$). Term tissues responded to LPS with a significant increase in PGE2 and numerical increase in PGF2α secretion, which were inhibited by FM ($p=0.00001$). These data indicate that placental tissues are capable of responding to the proinflammatory agent LPS through increased PG production. Interestingly, a differential secretion of PGF2α and PGE2 was seen in embryonic tissues, but not term tissues in response to LPS. This may signify selective Toll-like-receptor expression in the conceptus during early gestation, and is the first such report in the horse. Flunixin meglumine effectively inhibited LPS-induced prostaglandin secretion in all trials.

Keywords: Equine, pregnancy loss, inflammation, flunixin meglumine

Acknowledgement

This research was funded through a NCSU CVM Research Grant

Reference

1. de Mestre AM, Bacon SJ, Noronha LE, et al: Characterisation of chorionic girdle trophoblast vesicles in vitro and in vivo. Anim Reprod Sci 2006;94:424-425.

Leiomyoma in the spermatic cord of a stallion

Robyn R. Wilborn,[a] Aime K. Johnson,[a] Ghislaine A. Dujovne,[b] Valeria Z. Albanese,[a] Fred J. Caldwell,[a] Laura Catharine Brittain[a]

[a]Department of Clinical Sciences, College of Veterinary Medicine, Auburn University, AL; [b]Department of Population Health and Reproduction, College of Veterinary Medicine, University of California, Davis, CA

A 19 year old American Quarter Horse stallion presented for a routine breeding soundness examination (BSE). During examination, a palpable left paratesticular mass was appreciated measuring 4.5cm in diameter. The mass was non-painful and located in the area of the spermatic cord dorsal to the left testicle. A bilateral hydrocele was noted on ultrasonography but was minimal (2 mm) and unlikely to be clinically significant. The remainder of the BSE was within normal limits at that time. Without apparent effects on semen quality or breeding ability, the owner elected to monitor the mass and no further diagnostics were pursued. The stallion was successfully used for artificial insemination during the subsequent three breeding seasons with no apparent effect on fertility despite the continued presence of the lesion. Thirty months following initial examination, the patient presented for noticeable enlargement of the mass by the owner and overt bilateral hydrocele. The mass had increased to 10cm in diameter and profound hydrocele (2 cm) was apparent via visual inspection as well as digital palpation and ultrasonography. Ultrasonography also revealed significant dilation of the vessels in the left spermatic cord as compared to the right. To best preserve the patient's chance of remaining a breeding stallion, a left hemicastration was recommended. Surgical histopathology revealed clean excision of the mass, which was confirmed to be a leiomyoma. Leiomyoma of the testicle or associated structures is rarely reported in equids with only one previous case published. The leiomyoma in this case is suspected to have arisen from the tunica albuginea, which is composed of dense connective tissue as well as a smooth muscle cells. The mass presumably caused an increase in hydrostatic pressure in the vasculature of the spermatic cord, leading to a ten-fold increase in the hydrocele over a period of 2.5 years. Had the leiomyoma not been removed, semen quality would have undoubtedly declined due to inability to thermoregulate from the mass effect in the spermatic cord compounded by worsening hydrocele.

Keywords: Stallion, leiomyoma, spermatic cord, hydrocele

Selected references

Johnson RC, Steinberg H: Leiomyoma of the tunica albuginea in a horse. J Comp Pathol 1989;100:465-468.

Chacon-Arellano JT, Wooley DM: Smooth muscle cells in the testicular capsule of the horse, pig and sheep. J Anat 1980;131(Pt 2):263-273.

McEntee K: Reproductive pathology of domestic animals; 1990. San Diego: Academic Press. p. 279-306.

Experimental induction of nocardioform placentitis in mares

I.F. Canisso, B.A. Ball, M.H. Troedsson, A. Claes, K.E. Scoggin, E. Erol, N.M. Williams, E.L. Squires
Department of Veterinary Science, University of Kentucky, Lexington, KY

Nocardioform placentitis (i.e., infection caused by *Amycolatopsis* spp, and/or *Crossiela equi* (*C. equi*) or other actinomycetes) is associated with abortion and premature delivery in mares. However, the pathogenesis is unknown, and development of an experimental model for the disease is necessary. We hypothesized that inoculation of mares with *C. equi* can induce nocardioform placentitis. The study objectives were to determine whether inoculation of *C. equi*: intrauterine (IU) at 24 h post-breeding, or intravenous (IV), oral (PO), nasopharyngeal (NP) inoculation or intracervical (IC) administration between 180 and 240 d of gestation would result in nocardioform placentitis. Twenty mares received IU inoculation with 10^4 cfu of *C.equi* 24 h post-breeding with fresh extended semen without antibiotics. Endometrial samples were obtained 24 h after inoculation for PCR detection of *C. equi* DNA. Mares were monitored for placental disease by transrectal ultrasonography (15, 25, 35, 60, 90, and 120 d of gestation), and thereafter monthly by transabdominal ultrasonography until foaling. In addition, 15 pregnant mares were inoculated with 10^9 cfu of *C.equi* through NP (n=5), PO (n=4), IV (n=4) or IC (n = 2) routes from 180 to 240 d of gestation. NP and PO inoculations were administered four times every two weeks, whereas IV and IC inoculations were administered once, except one IC mare was re-inoculated two weeks later. Mares were examined with transabdominal ultrasonography bi-weekly from inoculation to foaling. A group of contemporaneous pregnant mares (n=60) kept on the same farm served as controls for all treated mares. For IC mares, transrectal ultrasonography was performed before inoculation and every other day for two weeks or until abortion, whichever came first. Placentas from all inoculated mares and ten placentas from the control group underwent complete pathologic evaluations and PCR for *C.equi* and *Amycolatopsis* ssp. Remaining control placentas were examined for gross signs of placentitis at the farm. *C. equi* was identified by PCR in 15% of mares (3/20) following IU inoculation, and the pregnancy rate was 95% (19/20 mares). Subsequently, two embryonic losses and one abortion at 177 days occurred, but the cause for these losses was not determined. Sixteen of the twenty mares delivered normal foals and placentas without signs of placentitis. In mares inoculated during pregnancy (i.e., 180-240 d), one mare in the oral administered group aborted at 200 days of gestation without an identifiable cause. The remaining 12 mares delivered normal, healthy foals and placentas. In the control group, one placenta presented lesions consistent with nocardioform placentitis, but PCR and cultures failed to identify *C.equi* or *Amycolatopsis* spp. For the IC group, one mare developed signs of ascending placentitis by 9 d after inoculation, and aborted 12 d post-inoculation. The remaining mare was re-inoculated 15 d later in a similar manner and she developed signs of placentitis 3 d later and aborted 7 d after inoculation. Both placentas and fetuses were negative on PCR for *C. equi* and pathologic evaluation revealed ascending placentitis caused by β-hemolytic *Streptococci*. In conclusion, we were unable to induce nocardioform placentitis in mares with inoculation of *C. equi* through different routes (ie IU, NP, PO, IV, IC). These findings bring into question the pathogenicity of the organism as well as the mechanisms required to establish nocardioform placentitis.

Keywords: Pregnancy loss, abortion, placental disease, pregnancy wastage, *Crossiela equi*

Acknowledgments
Funds were provided by the Kentucky Thoroughbred Association, Geoffrey Hughes Fellowship and the Albert G. Clay Endowment in Equine Reproduction, University of Kentucky.

Utilizing a wound healing assay to study canine trophoblast physiology in vitro

Justine M. Gullaba, Michelle A. Kutzler
Department of Animal and Rangeland Sciences, Oregon State University, Corvallis, OR

Introduction

The wound healing assay is used to study a cell's ability to proliferate and migrate in culture.[1] Interleukin-8 (IL8) and tissue inhibitor of metalloproteinase-2 (TIMP2) stimulate and inhibit, respectively, term human trophoblast proliferation and migration in vitro.[2,3] We hypothesized that term canine trophoblasts (TCTs) would respond similarly. The objectives were to compare wound healing rates in TCTs at increasing concentrations of IL8 and TIMP2.

Methods

Chorioallantois was collected from four dogs following term elective cesarean sections. Term canine trophoblasts were isolated from the chorioallantois as previously described[4] and seeded ($1X10^6$ cells/well) in 24-well plates in DME/H-21 medium (Gibco, #11965, Life Technologies, Grand Island, NY) supplemented with 10% FBS (Atlanta Biologicals, L11061, Lawrenceville, GA). Term canine trophoblasts were cultured at 37°C with 5% CO_2 until confluent. Cell proliferation and migration was studied using the wound healing assay as previously described.[1] The effects of recombinant human IL8 (#200-08M, Peprotech, Rocky Hill, NJ) and recombinant human TIMP2 (#410-02, Peprotech) were evaluated at 0, 10, 50, 100 ng/mL or 0, 0.05, 0.1, 0.5 µg/mL, respectively. The wound was photographed at 100X phase-contrast magnification with a digital camera (Fujufilm Fine AX300 14 MP, Tokyo, Japan) at 0, 8, and 12 hours of incubation. The wound area was measured using ImageJ v.1.34 software (http://imagej.nih.gov/ij/). The wound width at t=0 h was defined as 0% wound closure. Experiments were performed in triplicate. Data were analyzed by repeated measures ANOVA using SAS (Version 9.2, SAS Institute Inc., Cary, NC) in PROC MIXED. Results were summarized as mean±SEM % wound closure. Significance was defined as $p<0.05$.

Results

Compared to controls, wound healing rates were promoted with IL8 (10 and 50 ng/mL) at t=8 h (p=0.0172 and 0.0046, respectively) and 12 h incubation (p=0.0133 and 0.0005, respectively) and were reduced with TIMP2 (0.5 µg/mL) at t=8 h (p=0.0097) (Figure).

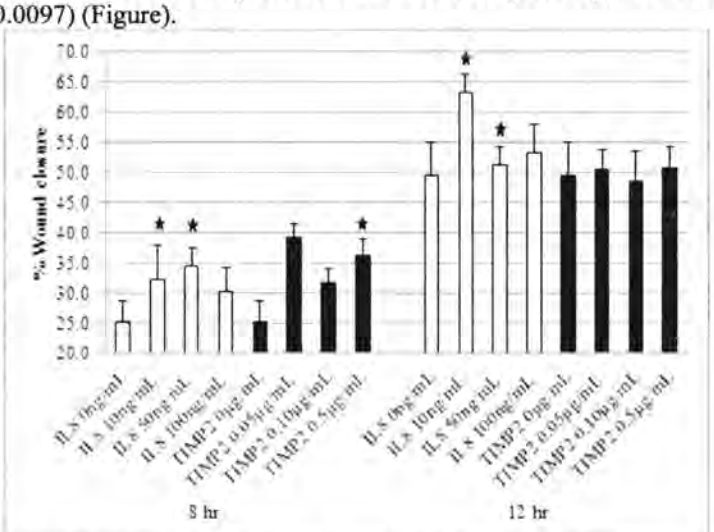


Figure. Effect of IL8 (open bars) or TIMP2 (solid bars) on wound healing in TCT (*p<0.05).

Discussion

An understanding of normal TCT physiology will provide a basis for research on abnormal TCT conditions (e.g. placental retention or subinvolution of placental sites). Additional investigations are underway to determine if differences in wound healing result from effects on migration, proliferation or both.

References

1. Liang CC, Park AY, Guan JL: In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat Protoc 2007;2:329-333.
2. Jovanovic M, Stefanoska I, Radojcić L, et al: Interleuken-8 (CXCL8) stimulates trophoblast cell migration and invation by increasing levels of matrix metalloproteinase (MMP)2 and integrins alpha5 and beta1. Reproduction 2010;139:789-798.
3. Lash GE, Otin HA, Innes BA, et al: Inhibition of trophoblast cell invasion by TGFB1, 2, and 3 is associated with a decrease in active proteases. Biol Reprod 2005;73:374-381.
4. Sahlfeld LM, Hazzard T, Kutzler M: Isolation and primary cell culture of canine trophoblasts [abstract]. Clin Therio 2011;3:358.

Effects of pyrethroid insecticides on cattle fertility

H.M. French,[a] F.A. Ireland,[b] C.F. Shipley,[a] V.L. Jarrell,[b] A.J. Fuselier,[a] D.G. Williams,[a] D.W. Shike[b]
[a]College of Veterinary Medicine and [b]Department of Animal Sciences, University of Illinois, Urbana, IL

Pyrethroid insecticides are commonly used in the beef cattle industry. Research with laboratory animals and case studies with livestock have suggested that these chemicals may reduce fertility. The objective of the following experiments was to determine if pyrethroids, cyfluthrin and beta-cyfluthrin, have negative effects on cattle fertility. In first experiment, Angus x Simmental bulls (n=24) ranging from one to six years of age (BW = 773.00 +/- 185.51 kg) were stratified by age and allocated to the following treatment groups: control (CON; n = 5), pour-on (POUR; n = 5), fly tag (TAG; n = 7), and pour-on and fly tag (POUR+TAG; n = 7). POUR bulls received 24 mL of 1% cyfluthrin pour-on (CyLence®, Bayer Animal Health, Shawnee, KS) by topical syringe, TAG bulls were ear tagged with two 8% beta-cyfluthrin (CyLence Ultra®, Bayer Animal Health) fly tags, and POUR+TAG bulls received both pour-on and fly tag as per label instructions. Bulls were maintained by treatment group on non-adjacent pastures and semen was collected weekly for nine weeks. Semen was analyzed for overall motility, progressive motility, and morphology with the aid of computer-assisted semen analysis. Blood samples were also taken weekly from the tail vein for analysis of testosterone. All data were analyzed in MIXED procedure of SAS. There were no differences in overall motility ($P = 0.41$), progressive motility ($P = 0.60$) or in normal morphology percentages ($P = 0.41$) among treatments. The testosterone concentration did not differ ($P = 0.16$) between control and treated bulls. In the second experiment, Angus and crossbred cows (n = 123) were blocked by breeding date (April and July) and by breed, and randomly assigned to a control (CON; n = 61) or treatment group (POUR+TAG; n = 62). The POUR + TAG group received both pour-on and fly tag at label doses. Cows were synchronized with a seven day CO-Synch+CIDR® program and bred by timed artificial insemination (AI) 66-72 hours from CIDR removal. Insecticide was applied to the treatment group at the time of CIDR insertion. Blood samples were collected via jugular venipuncture on day 10 and 17 after insemination to evaluate progesterone concentrations. Pregnancy evaluation was performed by rectal palpation in combination with real-time ultrasound at 35 days after insemination. All data were analyzed in MIXED procedure of SAS except for pregnancy data which were analyzed using the GENMOD procedure of SAS. The treatment group had decreased (P = 0.03) progesterone concentrations at day 10 when compared to control cows (5.51 vs. 6.40 ng/ml). Progesterone concentrations did not differ (P = 0.94) at day 17. No differences (P = 0.65) were observed in AI pregnancy rates between treated cows (45%) and control cows (40%). The pyrethroid fly tags and topical pour-on used in these studies, when used according to label instructions, did not adversely affect cattle fertility.

Keywords: Pyrethroid, insecticide, fertility, reproduction, cattle

GnRH immunization for the treatment of urinary incontinence in spayed bitches

C.E. Donovan,[a] J.M. Gordon,[b] M.A. Kutzler[a]
[a]Department of Animal and Rangeland Sciences, Oregon State University, Corvallis, OR; [b]College of Veterinary Medicine, Oregon State University, Corvallis, OR

Introduction

Urinary incontinence is common in spayed bitches (4.9-20%).[1,2] It has been postulated that permanently elevated gonadotropin concentrations as a result of ovariectomy may contribute to the development of this condition.[3] Therefore, the purpose of this study was to determine whether or not a gonadotropin releasing hormone (GnRH) vaccine, labeled for the management of benign prostatic hyperplasia in intact male dogs, would also be effective for the treatment of urinary incontinence in spayed bitches. It was hypothesized that spayed incontinent bitches immunized against GnRH would develop antibodies that would prevent gonadotropin elevation and permit a return to continence.

Methods

Nine privately-owned bitches with urinary incontinence following ovariohysterectomy were used. All bitches had been receiving daily oral phenylpropanolamine (PPA; Proin®, PRN Pharmacal, Pensacola, FL) to control incontinence prior to the start of the study. Six bitches received two subcutaneous injections of Canine Gonadotropin Releasing Factor Immunotherapeutic® vaccine (Pfizer Animal Health, Exton, PA) at four week intervals, and three bitches received two placebo injections at four week intervals. Venous blood samples were collected pre-vaccination (week 0) and 4, 6, 8, 10, 12, 16, 20, and 24 weeks post-vaccination. Vaccinated bitches discontinued PPA use two weeks after the second injection; control bitches remained on PPA for the duration of the study. Owners recorded all episodes of incontinence. Serum GnRH antibody titers were determined by an enzyme linked immunosorbent assay and antibody titers were analyzed using Fisher's exact test (GraphPad QuickCalcs Software, La Jolla, CA). Significance was defined as $p<0.05$.

Results

Side-effects of the vaccine included soreness (n=1) and swelling (n=1) at the injection site, lameness (n=1), and tachypnea (n=1, dog removed from the study). All side-effects resolved without treatment within 24 h. All dogs were seronegative for GnRH antibodies at week 0 and control dogs remained seronegative for the duration of the study. All vaccinated dogs developed GnRH antibody titers that were significantly higher than those of control dogs at weeks 4-16 ($p=0.02$). Of the five vaccinated dogs that completed the study, two dogs remained continent for 112.5±23.3 days after PPA was discontinued, while three dogs became incontinent 3.6±2.1 days after PPA was discontinued.

Conclusion

The pathophysiology of urinary incontinence following ovariectomy is not well understood. The results of this study show that GnRH immunization (and presumed decreases in gonadotropin concentrations) may be necessary but not sufficient to restore continence in all bitches. Plasma luteinizing hormone measurements are currently being performed to determine whether or not GnRH immunization actually resulted in decreased gonadotropin concentrations.

References

1. Arnold S, Arnold P, Hubler M, et al: Urinary incontinence in spayed bitches: prevalence and breed predisposition. Eur J Companion Anim Pract 1992;2:65-68.
2. Thrusfield MV, Holt PE, Muirhead RH: Acquired urinary incontinence in bitches: its incidence and relationship to neutering practices. J Small Anim Pract 1998;39:559-566.
3. Arnold S, Hubler M, Reichler I: Urinary incontinence in spayed bitches: new insights into the pathophysiology and options for medical treatment. Reprod Domest Anim 2009;44(Suppl 2):190-192.

Association of reproductive tract scoring of beef heifers and reproductive efficiency in AI and natural service beef herds

Katie Gutierrez, Ram Kasimanickam, Ahmed Tibary
Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, WA

Reproductive tract scoring is a method by which a numerical score from 1 (immature and acyclic) to 5 (mature and cyclic) is assigned to developing heifers based on the maturity of the ovaries and uterus, and cyclicity. This method can serve as a tool to eliminate heifers that may fail to breed. The objective was to determine the influence of reproductive tract score (RTS) on reproductive performance of beef heifers in beef operations using artificial insemination (AI) and clean-up (AI-CU) bulls or using natural service (NS) bulls in the breeding programs. The hypothesis was that heifers with higher RTS will have better reproductive performance. Angus-cross beef heifers (N=2,660) in AI-CU group were artificially inseminated at a fixed time (CO-Synch®+CIDR protocol) once then submitted to clean-up bulls two weeks later (bull:cow ratio=1:40 to 1:50) for the reminder of the 85 day breeding season. Angus-cross beef heifers (N=1,381) in NS group were submitted to bulls (bull:cow ratio=1:20 to 1:25) for the entire 85 day breeding season. Heifers received RTS four weeks prior to and body condition score (BCS; 1-emaciated; 9-obese) at the beginning of the breeding season. Pregnancy diagnosis was performed 70 days after AI and two months after end of breeding season for both AI-CU and NS breedings. Heifers in both groups were well managed and of similar age (M±SEM=14.9±0.4 [AI-CU] and 14.7±0.8 [NS] mo). Glimmix and Lifetest procedures of SAS were used to determine differences in the pregnancy rates (PR) and time to pregnancy during the breeding season, respectively. The PR for the AI breedings of the AI-CU group were different for heifers with different RTS ($P<0.0001$; 40.7, 48.3, 57.6 and 64.6% for RTS ≤2, 3, 4 and 5, respectively) after accounting for, BCS ($P=0.07$), heifer expressed estrus ($P=0.03$), year ($P=0.05$) and BCS by year interaction ($P=0.03$). Heifers with different RTS in the AI-CU group had different PR ($P<0.01$; 81.2, 86.5, 90.4 and 95.2% for RTS ≤2, 3, 4 and 5, respectively) after accounting for BCS ($P<0.05$) and year ($P<0.05$). Similarly, heifers different RTS in the NS group had different PR ($P<0.01$; 79.7, 84.3, 88.4 and 90.2% for RTS ≤2, 3, 4 and 5, respectively) after accounting for BCS and year ($P<0.05$). Heifers with higher RTS in both groups became pregnant earlier in the breeding season compared to heifers with lower RTS (log-rank statistics: $P<0.0001$; Table). Heifers in AI-CU group become pregnant at a faster rate compared to their counterparts in NS group ($P<0.01$).

Table. Median number of days* to pregnancy (25^{th}, 75^{th} percentile) along with the percentage of open cows by reproductive tract score (RTS) for heifers enrolled in the study.

Reproductive Tract Score	AI-CU[§] group			NS[♣] group		
	N (%)	% NP[⊗]	Median days (25^{th}, 75^{th})	N (%)	% NP[⊗]	Median days (25^{th}, 75^{th})
≤ 2	108 (4.1)	18.8%	28.5 (25, 55)	72 (5.2%)	20.3%	60 (34, 84)
3	596 (22.4)	13.5%	25 (10, 55)	283 (20.5%)	15.7%	60 (31, 84)
4	736 (27.7)	9.6%	10	370 (26.8%)	11.6%	53 (28, 75)
5	1220 (45.9)	4.8%	10	656 (47.5%)	9.8%	37 (26, 63)

*Kaplan-Meier product-limit method; [§]Heifers bred by artificial insemination followed by clean bulls; [♣]Heifers bred by natural service bulls; [⊗]Open heifers at the end of 85-d breeding season;

In conclusion, RTS was associated with both time to pregnancy and pregnancy rates. Further, heifers in natural service herds took more time become pregnant than heifers in AI-CU herds. This study indicates RTS can be used to assist in the selection of heifers that will conceive at a higher rate and breed earlier in the breeding season.

Keywords: Beef heifers, reproductive tract score, pregnancy rate, time to pregnancy

Laparoscopic-assisted ovariectomy in alpacas (*Vicugna pacos*)

L.K. Pearson, A.J. Campbell, A. Tibary
Comparative Theriogenology, Department of Veterinary Clinical Sciences, Washington State University College of Veterinary Medicine, Pullman, WA

Ovariectomy in alpacas may be indicated due to ovarian or oviductal abnormalities or for scientific investigation. The objectives of this study were to standardize a protocol for laparoscopic-assisted ovariectomy (LAO) which could be easily and reliably performed in alpacas, and to determine surgical steps which may offer difficulty in learning the technique. Fifteen mature alpacas were used. Five alpacas underwent LAO by experienced theriogenologists. Ten alpacas underwent LAO by fourth year veterinary students (two students per alpaca). Animals were anesthetized, placed in dorsal recumbency, and the abdomen surgically prepared. A 9 mm 30° endoscope was introduced through a portal at the umbilical scar after abdominal insufflation. A 5 mm instrument portal was placed off-midline in the caudal abdomen. The surgical procedure was divided into four steps and the time required to complete each step recorded. After animal placement in Trendelenburg position, the uterus was manipulated with laparoscopic forceps until the ovary was observed (Step 1). The ovary was then freed from the bursa (Step 2), grasped by the proper ligament, and elevated to the body wall on midline (Step 3). The animal was returned to a horizontal position and a 1.5 cm incision was made in the linea alba at the level of the elevated ovary. Allis tissue forceps were used to exteriorize the ovary; one transfixing ligature was placed using 0 polydioxanone and the ovary was transected from the pedicle (Step 4). Steps 1-4 were repeated with the contralateral ovary. The incisions were closed routinely. The effect of surgeon on time to complete each surgical step was examined using a one way ANOVA (Table). Students took significantly longer to perform steps 2 and 4.

Table. Time to completion (mean ±SEM, in minutes) of each surgical step by a theriogenologist or a student. Values with the different subscript within a row are statistically significant ($P<0.05$).

Surgical step	Surgeon			
	N	Theriogenologist	N	Student
Step 1	5	3.0 ± 0.7^{a}	10	3.4 ± 0.8^{a}
Step 2	10	3.0 ± 0.7^{a}	20	7.4 ± 1.8^{b}
Step 3	10	2.3 ± 0.5^{a}	20	2.7 ± 0.5^{a}
Step 4	10	6.8 ± 1.5^{a}	20	12.2 ± 2.3^{b}

In conclusion, LAO is a practical, minimally invasive technique in alpacas that can be easily learned. For students or practitioners preparing to perform the technique, emphasis should be placed on learning to manipulate the ovarian bursa to expose the ovary, exteriorization of the ovary, and placement of transfixing sutures, to decrease surgery and anesthesia times.

Keywords: Camelid, laparoscopy, ovary, surgery

Calcium carbonate in mammary gland secretions and fetal readiness for birth in alpacas

S. Skinner,[a] C. Fulton,[a] S. Holliday,[a] M. Jones,[b] D. Anderson,[b] V.C.L. Gomes,[c] M.S. Ferrer[b]
[a]Developing Scholars Program and [b]Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, Manhattan, KS; [c]CAPES Foundation Ministry of Education of Brazil, Brasilia, DF

Normal gestation length in alpacas is variable when timed from mating, and there are no reliable data to predict fetal readiness for birth. The objectives in this study were to determine if fetal readiness for birth could be predicted based on pre-partum concentration of calcium carbonate in mammary gland secretions and if daily sample collection affected passive transfer of immunoglobulin G (IgG). It was hypothesized that calcium carbonate concentration increases as parturition approaches, possibly in association with fetal readiness for birth, and that daily sample collection does not affect IgG transfer. Mammary gland secretions were collected daily by manual extraction for five days pre-partum (parturition = day 0) from eight pregnant female Huacaya alpacas. Five alpacas delivered normal crias (group NORMAL), while three delivered crias that died within 72 h of birth (group DIED). An equine stall-side calcium carbonate kit was used (FoalWatch K-1700, CHEMetrics, Calverton, VA) with a 1:12 dilution ratio of the sample. Plasma IgG concentration was measured in the crias 24 h after birth. Calcium carbonate concentration on day -1 and IgG concentration were compared between groups NORMAL and DIED with a T test. The sensitivity (100%), specificity (18%), positive (26%) and negative (100%) predictive values of a positive outcome (birth the day after sample collection) were calculated using ≥200 ppm as a positive response. The sensitivity (100%), specificity (58%), positive (22%) and negative (100%) predictive values were also calculated using ≥350 ppm as a positive response and birth on the day of sample collection as a positive outcome. Calcium carbonate concentration was ≥350 ppm in all samples on the morning of parturition, but some alpacas reached this value earlier (0 to -6 d). Also, calcium carbonate concentration was ≥200 ppm in samples from all alpacas on day -1 but some of them reached this value earlier (-1 to -9 d). No alpacas gave birth on a given day with concentrations <350 ppm or the following day with <200 ppm. Calcium carbonate concentration was higher on day -1 in alpacas in group NORMAL (281 ± 30 ppm; range 200 to 350 ppm) than in group DIED (100 ± 50; range = 50 to 150 ppm) (mean ± SE; P = 0.0071). Failure of passive transfer (IgG < 800 ng/mL) was diagnosed in one cria from each group. Plasma IgG concentration did not differ between crias in group NORMAL (1474.4 ± 416.1 ng/mL) and DIED (1651 ± 1549 ng/mL) (mean ± SE; P = 0.8760). It was concluded that concentration of calcium carbonate in mammary gland secretions on the day prior to parturition may allow prediction of neonatal survival and pre-natal identification of neonates that may need assistance. The test seemed to be a good negative predictor of parturition in alpacas. A calcium carbonate concentration <350 ppm or <200 ppm would provide a strong indication that parturition would not occur on that day or the following day, respectively. Daily sample collection did not affect passive transfer of IgG.

Keywords: Alpaca, parturition, calcium, fetal maturation, readiness for birth

Effects of early versus mid-diestrus $PGF_{2\alpha}$ administration in the mare

E.A. Coffman,[a] C.R.F Pinto,[a] H.K. Snyder,[a] C.A. Leisinger,[a] K. Cole[b]
[a]Department of Veterinary Clinical Sciences, The Ohio State University, Columbus, OH; [b]Department of Animal Sciences, The Ohio State University, Columbus, OH

In the present study, we hypothesized that serial administration of multiple doses of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to mares during early diestrus (beginning at day 0) will interrupt luteal function and induce a return to estrus similarly to mares treated with a single injection in mid-diestrus (day 10), and fertility of the induced estrus between the two groups will not differ. The specific objectives of the current study were to compare early and mid-diestrus $PGF_{2\alpha}$ treatment as assessed by: 1) determination of the duration of inter-ovulatory and treatment-to-ovulation intervals; 2) comparison of the number of pregnant mares at 14 days post-ovulation. Ten reproductively normal, cycling mares (median age 9 years, range 7-18) were examined daily by serial transrectal ultrasonography. In a balanced randomized crossover design, mares were assigned to one of two treatment groups at detected ovulation (day 0): I, mid-diestrus treatment, administration of a single 10 mg dose of $PGF_{2\alpha}$ (Lutalyse®, Pfizer Animal Health, New York, NY) im on day 10; II, early diestrus, administration of 10 mg $PGF_{2\alpha}$ im twice daily on days 0, 1 and 2 and once daily on days 3 and 4. Serial transrectal ultrasonography was performed to monitor follicular development throughout the treatment period. When a mare exhibited estrus and a follicle $\geq$35 mm in diameter was detected, she was artificially inseminated with $\geq$ 2 billion motile sperm from a fertile stallion and ovulation induced with 2500 IU human chorionic gonadotropin (hCG, Chorulon®, Intervet, Hillsboro, DE). Pregnancy was evaluated ultrasonographically 14 days after ovulation. Serial plasma samples were collected from all mares throughout the study to document the effects of $PGF_{2\alpha}$ treatment in early and mid-diestrus on luteal function. For all statistical analyses, significance was set at $P \leq 0.05$ and trend to significance as $0.05 < P \leq 0.10$. Data are expressed as mean ± SD. Inter-ovulatory and treatment to ovulation intervals were compared by paired t-test and fertility by McNemar's test. Progesterone data were analyzed by ANOVA for repeated measures (SigmaStat 3.5, Jandel Corp., San Rafael, CA). All mares in Group I underwent luteolysis after $PGF_{2\alpha}$ treatment, reflected by baseline mean±SD concentrations (0.25±0.21 ng/mL) of progesterone two days after treatment. The mean inter-ovulatory interval in Group I was 18.5 ± 2.0 days compared to 13.1 ± 3.7 days in Group II. In group II all mares experienced anti-luteogenesis following treatment with serial $PGF_{2\alpha}$ administration that resulted in mean±SD concentrations of progesterone to remain below 1.0 ng/mL. Treatment to ovulation intervals in Groups I and II were 8.5 ± 2.0 days and 13.1 ± 3.7 days, respectively ($P < 0.05$). In both Groups I and II, 9 of 10 mares became pregnant ($P = 1.0$). The results indicate that luteal function can be interrupted during early diestrus following serial administration of $PGF_{2\alpha}$ similarly to mares treated once with $PGF_{2\alpha}$ during mid-diestrus. Fertility of the $PGF_{2\alpha}$-induced estrus was not different in mares treated in early versus mid diestrus. Based on the present results, prevention of luteal formation does not affect fertility of the subsequent estrus.

Keywords: $PGF_{2\alpha}$, corpus luteum, equine, progesterone, luteolysis

Diagnosis and treatment of a gelding with seminal vesiculitis

A.J. Durand,[a] A.K. Johnson,[a] R. Funk,[b] R.R. Wilborn[a]
[a]Department of Clinical Sciences, College of Veterinary Medicine, Auburn University, Auburn, AL;
[b]Department of Clinical Sciences, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA

Seminal vesiculitis, although reported in stallions, is unusual in a gelding. A 14 year old Quarter Horse gelding presented with a one-year history of urine dribbling. Transrectal ultrasonographic examination revealed enlarged, fluid-filled seminal vesicles. Upon urethroscopic examination, the proximal urethra was found to be inflamed and the openings of the seminal vesicles were abnormally dilated.[1] Urine was collected by catheterization and the results of the urinalysis showed elevated protein (2990.0 mg/dL), red blood cells (20-50), white blood cells (50-100) and bacteria. Bacterial culture results identified *Citrobacter koseri.* Results of a complete blood count and serum chemistry were within normal limits. The gelding was treated with phenylbutazone (1 gram PO q 24 hours), trimethoprim sulfadiazine (20 mg/kg PO BID for 3 weeks) and discharged.

A follow-up urethroscopy was performed three weeks later, with no change in the findings. A luminal view within both seminal vesicles revealed inflamed walls and the presence of fibrin. A sample was taken for culture again identifying *Citrobacter koseri.* A five day treatment was instituted by lavaging both seminal vesicles with saline via urethroscopy once daily followed by an infusion of 500 mg amikacin buffered with an equal volume of sodium bicarbonate into each seminal vesicle.[2] Systemic antibiotic therapy was changed to enrofloxacin (5mg/kg IV q 24 hours) based on susceptibility. Following treatment, the seminal vesiculitis improved and there was minimal dribbling of urine at discharge.

To our knowledge, seminal vesiculitis in a gelding has not previously been reported. In stallions an infection in the seminal vesicles might compromise fertility by damaging the sperm or transferring infection to the bred mare. This gelding did not respond to conservative treatment, but did respond to lavages and targeted antibiotic therapy. Other options for treatment of chronic seminal vesiculitis include laser ablation of the glands and seminal vesiculectomy. It is possible that urethritis or cystitis led to the seminal vesiculitis observed in this patient.[3]

Keywords: Gelding, equine, seminal vesiculitis, urethritis

References

1. Varner DD, Blanchard TL, Brinsko SP, et al: Techniques for evaluating selected reproductive disorders of stallions. Anim Reprod Sci 2000;60-61:493-509.
2. Varner DD, Schumacher J: Abnormalities of the accessory sex glands. In: McKinnon AO, Squires EL, Vaala WE, et al, editors. Equine reproduction. 2nd ed. Ames(IA): Wiley-Blackwell; 2011. p. 1115-1117.
3. Furuya R, Takahashi S, Furuya S, et al: Is urethritis accompanied by seminal vesiculitis? Int J Urol 2009;16:628-631.

Use of a modified Vinsot technique for partial phallectomy due to paraphimosis

Jason Anton, Shelby Hayden, Reed Holyoak, Candace Jacobson
Veterinary Clinical Sciences, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK

Paraphimosis is a self-perpetuating medical condition characterized by marked edema of the internal preputial lamina of the penis due to impairment of venous and lymphatic drainage. Injury, priapism, phenothiazine administration, and severe debilitation are etiologies that may result in paraphimosis.[1] Techniques developed to correct paraphimosis vary from pharmacological intervention to salvage procedures. Frequently, surgery is indicated if the condition does not respond to medical treatment.[2-4] However, general anesthesia can prove problematic for debilitated patients and surgery tends to be cost-prohibitive for some owners.

A ten-year-old gelding was presented for paraphimosis with recurrent priapism resulting from an injury ten days prior. The erect penis was fully extended from an edematous sheath and showed visible signs of epithelial exfoliation upon presentation. The penis became partially flaccid after a 1% phenylephrine solution was injected into the corpus cavernosum, followed by lavage with heparinized lactated Ringer's solution. Application of a compressive bandage further reduced penile edema which allowed for penile repositioning and concurrent placement of a probang device.[3] Further evaluation revealed that the probang was causing discomfort due to the recurrent priapism; a sling was subsequently used to support the penis. Recommendation of a partial phallectomy and perineal urethrostomy (PU) were made due to the lack of resolution of the priapism and subsequent paraphimosis.

The modified Vinsot surgical technique, with a subischial perineal urethostomy, was chosen for the partial phallectomy. The PU was performed as previously described.[5] The modified Vinsot technique followed and was characterized by callicrate band placement proximal to the intended penile amputation site, penile amputation, and healing by second intention.[5]

The modified Vinsot technique is a standing procedure and practical alternative to previously described surgical techniques where general anesthesia is required. Furthermore, the cost for this particular technique can be significantly less and presents an affordable option to owners considering euthanasia.

References

1. Simmons HA, Cox JE, Edwards GB, et al: Paraphimosis in seven debilitated horses. Vet Rec 1985;116:126-127.
2. Scott EA: A technique for amputation of the equine penis. J Am Vet Med Assoc 1976;168:1047-1051.
3. Brinsko SP, Blanchard TL, Varner DD: How to treat paraphimosis. Proc Annu Conv Am Assoc Equine Pract; 2007. p. 580-582.
4. Van Harreveld PD, Gaughan EM: Partial phallectomy to treat priapism in a horse. Aust Vet J 1999;77:167-169.
5. Arnold CE, Brinsko SP, Love CC, et al: Use of a modified Vinsot technique for partial phallectomy in 11 standing horses. J Am Vet Med Assoc 2010;237:82-86.

Satisfactory semen quality after testicular rupture and hemicastration in a bull

V.C.L. Gomes,[a] L.M.J. Miller,[b] M.D. Miesner,[b] B.C. Fraser,[b] M.S. Ferrer[b]
[a]CAPES Foundation, Ministry of Education of Brazil, Brasilia, DF; [b]Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, Manhattan, KS

Hemicastration may allow preservation of fertility of valuable bulls after unilateral testicular and scrotal injury if performed early to prevent damage in the contralateral testicle.[1-3] This case report describes production of satisfactory semen quality in a bull after hemicastration due to testicular rupture and scrotal hematoma.

A 20-month old Red Angus bull was presented with unilaterally swollen scrotum noticed two weeks earlier. No other history was available. Fever, tachycardia and tachypnea were present. The left scrotum was painful, warm and enlarged. Differential diagnoses included scrotal hernia, hematoma, hematocele, pyocele, periorchitis, epididymitis and orchitis. On scrotal ultrasonography, the left hemiscrotum was thick with irregular borders. There were areas of anechoic fluid with hyperechoic fibrin strands within the vaginal cavity. No other scrotal contents were identifiable. These findings were consistent with scrotal edema and hematoma. Scrotal hematoma typically occurs secondary to trauma and blood vessel rupture within the spermatic cord or tunica albuginea. Treatment options include bilateral or unilateral castration. Prognosis for fertility after hemicastration depends on the functionality of the remaining testicle possibly affected by inflammation and impaired thermoregulation.[2] Moreover, anti-sperm antibody formation and immunoinfertility could occur after blood-testis barrier disruption.[4] Hemicastration was elected due to animal's genetic value and a grossly normal right testicle. The diagnoses of scrotal hematoma and rupture of the testicle and tunica albuginea were confirmed at surgery.

A breeding soundness evaluation was performed two months after surgery. Scrotal circumference was 35 cm. Total and progressive sperm motility were 95 and 82%, respectively, with 85% morphologically normal sperm. The percentage of IgG- and IgA-bound sperm was 0.62 and 1.26%, respectively. Semen quality was satisfactory after hemicastration despite the chronicity of the testicular rupture and hematoma. Immunoinfertility did not seem to be a complication of testicular rupture. Hemicastration seemed a viable treatment for this bull, allowing preservation of breeding potential.

References

1. Heath AM, Baird AN, Wolfe DF: Unilateral orchiectomy in bulls: a review of eight cases. Vet Med 1996;91:786,788-792.
2. Ivany JM, Anderson DE, Ayars WH, et al: Diagnosis, surgical treatment, and performance after unilateral castration in breeding bulls: 21 cases (1989-1999). J Am Vet Med Assoc 2002;220:1198-1202.
3. Wolfe DF, Hudson RS, Carson RL, et al: Effect of unilateral orchiectomy on semen quality in bulls. J Am Vet Med Assoc. 1985;186:1291-1293.
4. Srinivas M, Hashim S, Mitra DK: Unilateral blunt testicular trauma in pre-pubertal rats. Pediatr Surg Int 1999; 15:457-460.

Late term twin pregnancy management in a Thoroughbred mare

F.M. Whitehill, M.A. Pozor, M.L. Macpherson, L. Husted, L. Dillon, A.A. Kelleman, M.M. Diaz
Large Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL

Late term twin pregnancy in mares is undesirable due to poor fetal/neonatal survival and high risk of periparturient complictions.[1] Most third trimester twin pregnancies are spontaneously aborted.[2,3]

A 15-year-old Thoroughbred mare was presented at the University of Florida College of Veterinary Medicine at day 250 of gestation for precocious mammary development and suspected fetal death. Examination of the mare revealed an enlarged mammary gland and an elongated vulva. Transrectal examination of the reproductive tract revealed abnormal presentation of fetal membranes suggesting placental compromise. Transabdominal ultrasound examination revealed one viable and one non-viable fetus of similar sizes. Pregnancy termination was initiated due to the risk for premature delivery and/or dystocia. Parturition was induced using low dose (5 IU) oxytocin. Both fetuses were extracted manually after intramuscular injection of oxytocin. The first fetus was delivered dead and the second was euthanized. The mare spontaneously passed both placentas. The mare was examined daily by transrectal ultrasound to assess uterine involution. The mare was treated every six hours with intramuscular injections of oxytocin to aid in uterine contraction. Uterine lavage was performed daily for three days and effluent was within normal limits. Pentoxiphylline, flunixin meglumine, trimethoprim sulfa and light exercise were prescribed. She was discharged three days after the abortion, and did well after discharge.

Rarely, early detection methods (including transrectal ultrasonography) miss twin pregnancies. Early intervention techniques (manual reduction, fetal reductions) reliably result in delivery of a single foal.[4] Late gestational twin pregnancies frequently result in abortion due to the epitheliochorial type of placentation.[4] Twins that survive late into gestation are at high risk of premature delivery and dystocia.[2] Live born twins are often dysmature and small, with a high rate of mortality.[3] Given the inherent risks, cost and chance of survival of one foal, termination of the pregnancy is often performed.

References

1. Blanchard TL, Varner DD, Schumacher J: Manual of equine reproduction. 2nd ed. St. Louis: Mosby; 2003.
2. Ginther OJ, Griffin PG: Natural outcome and ultrasonic identification of equine fetal twins. Theriogenology 1994; 41:1193-1199.
3. Jeffcott LB, Whitewell KE: Twinning as a cause of foetal and neonatal loss in the thoroughbred mare. J Comp Pathol 1973;83:91-106.
4. McKinnon AO: Twin reduction techniques. In: Samper JC, Pycock JF, McKinnon AO, editors. Current therapy in equine reproduction. St. Louis: Saunders Elsevier; 2007. p.368, 371-372.

A case of female pseudohermaphroditism in a Dorset cross sheep

Sara Steidl, Bret McNabb, Catalina Cabrera
Livestock Reproduction and Herd Health, Veterinary Medical Teaching Hospital, School of Veterinary Medicine, University of California-Davis, Davis, CA

A two- month-old Dorset cross ram presented to the University of California-Davis Veterinary Medical Teaching Hospital for evaluation and treatment of a scrotal hernia and castration. Palpation of the scrotum did not reveal the presence of testicles but on ultrasound examination the scrotum appeared to be filled with loops of bowel. Surgical exploration revealed the contents of the scrotum were not bowel but a portion of intact uterus and ovaries. The uterus was replaced, the inguinal rings closed, and scrotal ablation was performed. The lamb was allowed to grow until eight months of age when it was harvested.

At the time of slaughter the animal was small in stature compared to the rest of its cohorts and maintained a juvenile-sized penis. Swelling of the ventral peri-anal region was also noted. The urogenital tract and a muscle biopsy were collected for further evaluation. The tract had a complete uterus, with two fully functional ovaries and a blind-ended vagina. The bladder and urethra communicated with an infantile penis. The tract also contained what grossly appeared to be accessory sex glands.

Muscle samples used for genotyping revealed the animal had a 54XXgenotype, with no evidence of chimarism. Histopathology of the gonads found follicular development with no evidence of testicular tissue. At this point the lamb was considered to be a female pseudohermaphrodite. Female pseudohermaphrodism is a rare occurrence in sheep and other domestic species. The causes of female pseudohermaphrodism are often linked to hormonal changes in utero but may also be the result of chromosome changes. In this case, linking the genital abnormalities to a hormonal cause is difficult because this animal has a normal female twin. This is one of few reports of intersexuality in sheep not related to freemartinism.

Selected references

Cribiu EP, Chaffaux S, Durand V, et al: Cytogenetic, anatomical, histological and hormonal study of a female pseudohermaphrodite cow. Reprod Domest Anim 1989;24:265-273.
Lott DF, Benirschke K, McDonald JN, et al: Physical and behavioral findings in a pseudohermaphrodite American bison. J Wildlife Dis 1993;29:360-363.
Vallenzasca C, Galli A: Andrological study of an intersex ram. Andrologia 1992;24:345-348.
Smith KC, Long SE, Parkinson TJ: A case of true hermaphroditism in a sheep. Vet Rec 1996;138:497-498.
Bunch TD, Callan RJ, Maciulis A, et al: True hermaphroditism in a wild sheep: a clinical report. Theriogenology 1991;36:185-190.

Single mummified fetus in a mare

Ana K. Malone Oliver, Tamara Dobbie
Department of Clinical Studies, School of Veterinary Medicine, University of Pennsylvania, New Bolton Center, Kennett Square, PA

Mummification of an equine fetus, though rare, is more commonly associated with a twin pregnancy rather than singleton pregnancy.[1] A single mummified fetus was removed from the uterus of a 17-year-old multiparous Arabian mare at 341 days of gestation. Throughout the 11 month gestation, the mare was administered 22 mg altrenogest (Regu-Mate, Intervet Inc., Millsboro, DE) PO SID. When the mare's mammary gland failed to develop at 339 days of gestation, altrenogest was discontinued and the mare came into estrus.

Transrectal palpation and ultrasonography of the mare's reproductive tract revealed a closed cervix, moderate uterine edema, and a mummified fetus approximately 90-days of age in the uterine body. The fetal bones were mineralized and no fetal fluids remained. Aerobic culture of an endometrial swab obtained prior to uterine manipulations failed to produce any bacterial growth. The mare's cervix was digitally dilated under sedation; the mummified fetus and fetal membranes were manually removed. Following the procedure, the mare's uterus was lavaged with sterile saline and oxytocin was administered (20 IU, IV) for two consecutive days.

Reports of fetal mummification in the mare are rare and most are associated with twin pregnancy. Mummification typically occurs during the third to eighth month of pregnancy following the death of the fetus in a sterile environment.[2] Once a fetus dies in utero placental progesterone production ceases and the fetus is expelled from the uterus.[1] In the case of fetal mummification, the fetus is maintained in the mare's uterus where it undergoes autolysis and dehydration. The fetus is maintained by a continued source of progesterone[3] from either a functioning corpus luteum, an intact fetoplacental unit from a surviving twin, or from an exogenous source.[3] In this mare, oral progestogen supplementation (altrenogest) maintained cervical closure and prevented expulsion following death of the fetus.[4]

This case highlights the importance of regular fetal monitoring when mares receive progestogens during pregnancy. If a fetus dies in utero while a mare is receiving progestogen therapy, expulsion of the dead fetus may not occur leading to fetal mummification.[5]

References

1. Card CE: Fetal maceration and mummificaaton. In: McKinnon AO, Squires EL, Vaala WE, et al, editors. Equine reproduction. 2nd ed. Ames (IA): Wiley-Blackwell; 2011. p. 2373-2375.
2. Pizzigatti D, Batista FA, Martins CF, et al: Haematic mummification in a mare with twin pregnancy. J Equine Vet Sci 2012;32:305-308.
3. Newcombe JR, Wilson MC: Mummified singleton foetus in a mare. Aust Equine Vet 1998;16:33-34.
4. Barber JA: Mummified fetus in a mare. J Am Vet Med Assoc 1996;208:1438-1440.
5. McCue, PM, Vanderwall DK: Fetal mummification in a mare. J Equine Vet Sci 1997;17:267-269.

Immunolocalization of matrix metalloproteinase-2 and membrane type 1 matrix metalloproteinase in canine testes

Chuleekorn Warinrak, Feng-Pang Cheng
Department of Veterinary Medicine, National Chung Hsing University, Taichung 402, Taiwan

Matrix metalloproteinase-2 (MMP-2) activity is important for spermatogenesis. Membrane type 1 (MT1)-MMP controls proMMP2-activation in Sertoli cells. This study was designed to demonstrate immunolocalization of MMP-2 and MT1-MMP in canine testes. It was postulated that MT1-MMP and MMP-2 are localized in Sertoli cells and germ cells at various stages of spermatogenesis.

Testes from healthy dogs (N = 12; > 2 years of age) were sectioned and fixed in modified Davidson's solution for at least 24 h. Immunohistochemistry was performed and 10 fields per specimen were evaluated (400 ×) in a semi-quantitative manner for the analyses of immuno-reactivity of MMP-2 and MT1-MMP staining. Slides were scored as the estimated area in proportion (P; scale 0 to 4) and intensity of positive-staining cells (I; scale 0 to 4). The total score (TS) was the sum of P and I. For determination of MT1-MMP and MMP-2 gene expression, testicular samples were homogenized, purified, total RNA extracted, complementary DNA synthesized and PCR was performed.

Cytosol staining of MT1-MMP showed a significant distribution (TS=8) in Sertoli cells and spermatogonia, while no staining of MT1-MMP (TS=0) was observed in spermatocytes, spermatids and elongated spermatids. Cytosol staining of MMP-2 was concentrated mainly (TS=8) in the perinuclear region of round spermatids, representing the region of developing Golgi organelles. Interestingly, MMP-2 was located at the tips of elongated spermatids (TS=8) while spermatocytes and spermatogonia showed negative staining (TS=0). Both amplified amplicons of MT1-MMP and MMP-2 were detected by PCR and were observed on agarose gel electrophoresis. Similar results were found in specimens from all dogs.

In conclusion, this is the first report demonstrating the presence of MT1-MMP in Sertoli cells and spermatogonia, and MMP-2 in round and elongated spermatids in dogs.

Keywords: MT1-MMP; MMP-2, Sertoli cells, acrosome development, canine testis

Female rat reproductive anatomy and non-surgical embryo transfer

Marni Hershbain, Sandra L. Ayres
Tufts Cummings School of Veterinary Medicine, North Grafton MA

Our laboratory has been investigating the reproductive anatomy of the female rat as it relates to non-surgical embryo transfer. Although there are a number of references for the anatomy of the rat reproductive tract, there is disagreement among the various anatomical descriptions. We hypothesized that an accurate assessment of the rat reproductive tract would enable us to begin designing a method for transcervical embryo transfer in the rat. The purpose of this study was to perform dissections on rats of a variety of sizes and two breeds in order to accurately describe and make measurements of the rat reproductive tract (length of the vagina, length of cervix, length of uterine horns, length of the ovary + bursa). In addition, this study would provide useful reference information on female rat reproductive anatomy.

Anatomical measurements were taken of 39 Sprague Dawley and six nude female rats. Rats were obtained from other investigators at Tufts after use in unrelated studies and euthanized on the premises. Additional rats were obtained from other facilities after being euthanized and stored cold for same day dissection. Measurements were made using a digital caliper. For each specimen we recorded the length of the vagina, the length of the cervix, the length of the ovary + ovarian bursa, and the length of the uterine horns. To expose the reproductive tract we opened specimens from the floor of the vagina to the lower abdomen, also cutting through the pelvic girdle. Horns and ovaries were exposed and measured. We recorded weights for each specimen and took photographs of representative animals to illustrate the rat's unique anatomy.

Mean measurements for length of vagina, length of cervix, length of uterine horns (right and left), length of the ovary plus ovarian bursa (right and left) and mean body weight are shown below.

	Weight (g)	Length of vagina (mm)	Length of cervix (mm)	Length of uterine horns (R/L) (mm)	Length of ovary + Bursa (R/L) (mm)
Mean	**255.82**	**15.77**	**9.40**	**43.30/42.73**	**5.82/5.94**

Weight was significantly correlated with vaginal length, cervical length and ovary size (Pearson's correlation $p<0.05$). Weight was not correlated with uterine horn length, but this might be explained by the different life history of the rats used in this study, some of which had previously reproduced and some of which had not.

We found that rats have a single cervical os surrounded by four petal-like papilla (top, bottom, and either side). Although externally the rat appears to have one cervix, internally the cervix is immediately divided by a septum into two non-communicating canals. The septum continues to the bifurcation of the uterine horns, preventing the horns from communicating with each other.

Aside from serving as an anatomical guide, the findings presented here have a major implication for the further development of embryo transfer techniques in rats. Knowing that the uterine horns are not in communication necessitates that for both horns to be gravid, embryos must be introduced on both sides of the cervical septum. Taking these anatomical findings into account, further work is being done in our laboratory to develop a non-surgical embryo transfer technique for rats.

Keywords: Rat, embryo transfer, reproductive anatomy, rat cervix

Evaluation of canine sperm morphology using two techniques for sperm separation

Rachel Hegedus, Michelle Kutzler
Department of Animal and Rangeland Sciences, Oregon State University, Corvallis, OR

Introduction

Veterinarians often encounter subfertile male dogs whose poor quality semen is intended for artificial insemination. Several methods for the elimination of abnormal sperm are available. Density gradient centrifugation (DGCM) and the swim up method (SUM) have both been shown to be effective in separating sperm but their efficacy in recovery of morphologically normal canine sperm has not been compared. The objective of this study was to compare sperm morphology following these two methods of sperm separation. With the success and widespread use of commercially-available DGCM for horses,[1] we hypothesized that this method would yield a higher percentage of morphologically normal sperm than the SUM.

Methods

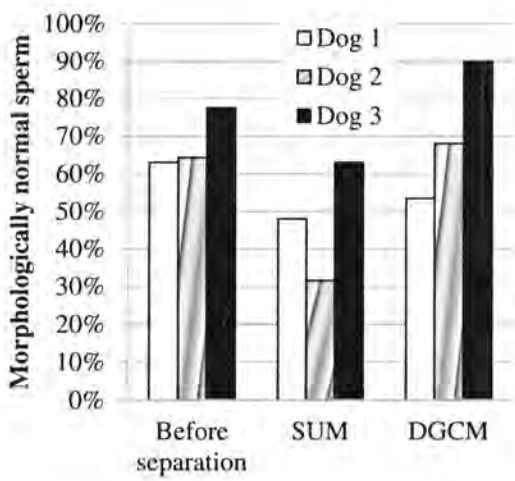
Semen was manually collected from three dogs who had sired a litter within a year from semen collection. Samples were divided into three aliquots of equal volume. Sperm morphology was assessed immediately prior to sperm separation using an eosin-nigrosin stain and by counting 200 sperm under oil immersion. For the DGCM, Equipure™ (Nidacon International, Mölndal, Sweden) was overlaid with the semen sample and centrifuged for 30 min at 100 x *g* as previously described for horses.[1] For the SUM, the semen sample was centrifuged twice for 15 min at 215 x *g* in Ham's F-10 and then incubated at 37°C in 5% CO_2 at a 45° angle for 60 min as previously described for humans.[2] Sperm morphology was again assessed following sperm separation. Using a paired Student's t test (Microsoft Office Excel 2007, Redmond, WA) the percent of morphologically normal sperm before and after each of the separation methods was compared. Significance was defined as $p<0.05$.

Results

There was no significant difference in the percent of morphologically normal sperm before separation and following DGCM. However, there was a trend towards a lower percentage of morphologically normal sperm in samples separated using the SUM (see figure below) than in fresh semen ($p<0.06$) and after DGCM ($p<0.09$).

Discussion

Equipure™ density gradient centrifugation is easier to perform than other DGCMs reported in dogs[3] because it only requires one overlay layer. In addition, there is no need to perform any additional washing steps after using this method of separation. Ongoing studies are comparing sperm RNA purity and yield between these methods.



References

1. Das PJ, Paria N, Gustafson-Seaberry A, et al: Total RNA isolation from stallion semen and testis biopsies. Theriogenology 2010;74:1099-1106.
2. Jameel T: Sperm swim-up: a simple and effective technique for semen processing for intrauterine insemination. J Pak Med Assoc 2008;58:71-74.
3. Dorado J, Alcaráz L, Duarte N, et al: Centrifugation on PureSperm® density-gradient improved quality of spermatozoa from frozen-thawed dog semen. Theriogenology 2011;76:381-385.

Global endometrial gene expression during simulated diestrus, estrus and anestrus in the bitch

N. Krekeler,[a] N.D. Young,[a] D.M. Noden,[b] G.F. Browning,[a] J.A. Charles,[a] P.J. Wright[a]
[a]Faculty of Veterinary Science, The University of Melbourne, Werribee, Victoria, Australia; [b]Cornell University, Department of Biomedical Sciences, Ithaca, NY

It has been shown in an *in vivo* canine pyometra disease model that the disease can only be induced reliably if *E. coli* is inoculated into the uterus during simulated diestrus; inoculation during simulated estrus or anestrus does not cause pyometra.[1] These observations raise the possibility that the uterine immune response may differ during different stages of the estrous cycle.

The objectives of this study were to characterize differences in host immune defences at different stages of the estrous cycle in order to elucidate the differences in disease susceptibility in relation to ovarian hormone concentrations.

Twelve post-pubertal, ovariectomized greyhound bitches were treated with estradiol benzoate (Intervet, Bendigo East, Victoria, Australia) and/or megestrol acetate (Jurox Pty Ltd., Rutherford NSW, Australia) to simulate estrus or diestrus. Uteri were obtained either on day 4 of simulated estrus or on day 10 of simulated diestrus (n=4 per group). Untreated animals served as anestrous controls (n=4). Punch biopsies of uterine tissue were preserved in RNAlater® (Applied Biosystems, Scoresby, Victoria, Australia) and total RNA was extracted, mRNA purified and randomly transcribed into cDNA. Pooled cDNA libraries from simulated diestrus, estrus or anestrus groups were paired-end sequenced using the Illumina platform (Illumina® Genome Analyzer II). Paired-end reads were mapped to the annotated canine genome (Broad Institute, Massachusetts Institute of Technology and Harvard University, Cambridge, MA). The total number of mapped reads was normalized for gene length and pair-wise statistical differences in gene expression were determined using a false-discovery rate corrected *P*-value of ≤ 0.01, a minimum 4-fold difference in expression and filtration for genes with more than 100 reads mapped in at least one treatment group.

Comparison of biopsies from uteri in simulated diestrus to those from uteri in simulated estrus identified 496 genes that were differentially expressed (264 genes were upregulated and 232 were downregulated in simulated diestrus). Comparison of biopsies from uteri in simulated diestrus to those from uteri in simulated anestrus revealed 708 differentially expressed genes (325 were upregulated and 383 downregulated in simulated diestrus). Several of the most highly up- and downregulated genes play a role in innate immunity, including beta-defensins, aquaporins, cadherins, lactoferrin, serum amyloid A and alpha-antitrypsin.

In conclusion, cycle stage-specific uterine tissues revealed pronounced differential gene expression, suggesting that ovarian hormone profiles play an important role in the pathogenesis of canine pyometra.

Keywords: Dog; pyometra; RNA-Seq; estrous cycle

Reference

1. Arora N, Sandford J, Browning GF, et al: A model for cystic endometrial hyperplasia/pyometra complex in the bitch. Theriogenology 2006;66:1530-1536.

The effects of method and operator experience on the accuracy of canine sperm counts

Robyn R. Wilborn,[a] Kaitlyn N. Caraway,[a] Aime K. Johnson,[a] Jay Z. Barrett,[b] Todd D. Steury[c]
[a]Department of Clinical Sciences and [b]Animal Health and Performance Program, College of Veterinary Medicine; [c]School of Forestry and Wildlife Sciences, Auburn University, Auburn, AL

Accurately measuring the concentration of spermatozoa in canine semen is of paramount importance as more veterinary practices offer semen evaluation, shipping and cryopreservation. Challenges commonly encountered when determining sperm concentration include cost of equipment, limited time, and lack of properly trained staff members to perform the procedure. The perfect quantification method for clinical use would be accurate, cost-effective, efficient and easy to perform. We hypothesized that the experience level of the operator would significantly impact the accuracy of the commonly used methods for measuring sperm concentration.

The improved Neubauer hemocytometer (HEMO) has long been considered the gold standard for the determination of sperm concentration in semen samples. While this is a cost-effective option, the process is tedious, time-consuming and presumably requires an experienced operator. This study compared the results obtained with the HEMO to those obtained with three commercially available methods, each representing a different cost and method of quantification. The commercially available methods used were the 591B Densimeter (DENS; Animal Reproduction Systems, Chino, CA), NucleoCounter® SP-100 (NC; Chemometec, Allerod, Denmark), and SpermVision SAR® computer assisted sperm analysis system (CASA; Minitube of America, Inc., Verona, WI). One hundred semen samples were obtained from a total of 18 healthy dogs of varying breeds over a three month period. Each sample was analyzed in duplicate by an experienced and inexperienced operator, using each of the four methods. The inexperienced operator was trained for a total of two hours among all methods. During this time, hands-on instruction was provided and two practice samples were counted for each method which were not included in the analyzed data. Proper procedure for each method was also posted in the laboratory so that the inexperienced operator could quickly refer to the protocols at any time, but no further hands-on instruction was provided.

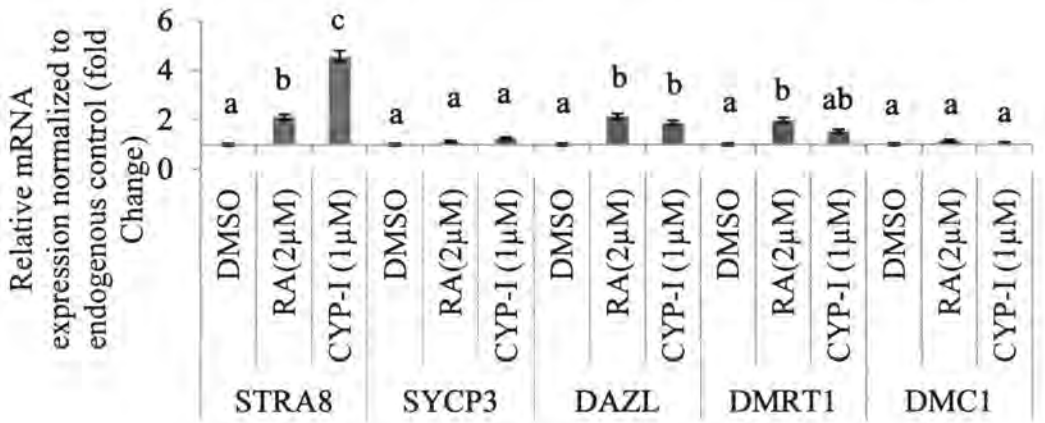
Using a linear mixed-effects model, all three methods (DENS, NC, CASA) generated significantly biased estimates of concentration compared to HEMO ($F_{3,697}$=86.05884; p<0.0001). However, results indicated that the experience level of the operator did not influence the accuracy of any of the methods, as neither the main effect for experience ($F_{1,693}$=0.15; p=0.70), nor the interaction between experience and method ($F_{3,693}$=2.19; p=0.09), were statistically significant. Similarly, neither the interaction between sample number (i.e. experience gained over time) and experience, nor the interaction between sample number, experience, and method were significant ($F_{1,686}$=0.48184, p=0.4878; and $F_{3,686}$=0.36027, p=0.7817, respectively) indicating that the effects of experience on accuracy of methods were not influenced by the number of samples. Thus, experience level of the operator did not have a significant impact on accuracy for any of the methods tested.

Keywords: Spermatozoa, canine, concentration, accuracy, hemocytometer

Exogenous and endogenous retinoic acid modulates meiosis-associated genes expression in canine testis, an *in-vitro* model

Vanmathy Kasimanickam, Ramanathan Kasimanickam
Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, WA

Pharmacological approaches to the control of spermatogenesis in dogs are required for the long-term solution to the population explosion amongst dogs. The objectives were 1) to investigate the regulation of meiosis-associated and male germ cell-related genes (STRA8, SYCP3, DMC1, DMRT1 and DAZL) following exogenous administration of retinoic acid (RA) and after modulation of endogenous RA by a CYP26B1 inhibitor in an in-vitro canine testis model; and 2) to compare the effect of increased endogenous RA by inhibition of the enzyme CYP26B1 to the consequences of direct administration of exogenous RA. Testes of five healthy, medium-sized dogs of mixed breed were used for the organotypic cultures. The dogs were about seven months old. Testes cultures were carried out following a previously described procedure. All *trans*-RA at 2μM final concentration, CY26B1 inhibitor R115866 at 1μM final concentration and the control, dimethyl sulphoxide (DMSO) were administered to the testes cultures and the cultures were maintained for 24 h. Subsequent to these *in-vitro* treatments, real time PCR was performed to analyze the meiosis-associated genes expressions in the testis by ANOVA using $2^{-\Delta\Delta Ct}$ values to ascertain statistical significance of any differences in gene expressions (see figure).



abc - Different supercripts within gene are different ($P<0.05$)

Genes STRA8, DAZL and DMRT1 were significantly up-regulated as a result of the direct and indirect increase of RA in the testis, after the exogenous administration of all trans RA and CYP26B1 inhibitor. Up-regulation of STRA8 was very prominent compared to DAZL and DMRT and the drastic up-regulation of STRA8 was also observed with CYP26B1 inhibitor. Since DAZL encodes a germ cell-specific RNA binding protein, required for the induction of STRA8 and initiation of meiosis, we might see the expression differences temporally with the stage of spermatogenesis. DMRT1 is a unique gonad and stage specific transcription factor, directly activates STRA8 and has a temporal influence on its expression. No significant differences were found with the early meiotic markers, SYCP3 and DMC1 with RA, CYP26B1 inhibitor and vehicle treatments. In conclusion, pharmacological intervention of canine spermatogenesis pertinent to RA signaling is plausible and the effect of modulation of spermatogenesis differs upon the types of pharmacological targets such as agonists, antagonists and inhibitors. Temporal and spatial influences on spermatogenesis should also be considered.

Keywords: Dog, spermatogenesis, meiosis-associated gene, retinoic acid, CYP 26B1

Ultrasonographic estimation of gestational age in bitches of various sizes

Jo L. Randall,[a] Jay L. Randall,[a] J.P. Kastelic,[b] M.C. Windeyer[b]
[a]Animal Hospital of Woodstock, Woodstock, IL; [b]Department of Production Animal Health, University of Calgary, Calgary, Alberta, Canada

There are good formulas to estimate canine fetal age, but most are not user-friendly. Following preliminary observations, the objective of this study was to determine the potential of using the inner chorionic cavity (ICC) to crown-rump length (CR) ratio as a quick and reliable estimate of gestational age in dogs. Forty-seven purebred bitches of various sizes, and 24 to 44 days post-ovulation (plasma progesterone concentrations 4.0 to 6.0 ng/ml) were used. The weight and size of each bitch were recorded as small (< 11.3 kg), medium (11.3 - 22.6 kg), large (22.7 - 45.3kg), or giant (≥45.4 kg). Bitches were placed in dorsal recumbency and a 3-9 MHz ultrasound transducer (Esaote My Lab Class C, Esaote North America, Indianapolis, IN) was used to produce the following images: 1) transverse view of a circular ICC; vertical and horizontal diameters were measured and averaged; and 2) sagittal view of the fetus to measure maximal CR. Up to three fetuses per bitch were measured and the mean determined for each of the two end points, ICC and CR. The ICC to CR ratio (ICC_x/CR_x) was calculated for each fetus and for each bitch (=ICC/CR_{ave}; mean of all fetuses). Associations between gestational age and the potential explanatory variables (ICC, CR, and ICC/CR) were assessed in multivariable linear regression models (PROC GLM, SAS 9.2, SAS Institute, Cary, NC). Potential covariates (bitches' weight, age, and size category [SIZE]) that were significant at $P < 0.10$ in univariable screening were offered to the multivariable models and removed by backwards elimination when $P > 0.05$. Interaction terms between remaining variables were tested. Residuals were evaluated to determine if transformation was necessary. The quadratic term for ICC/CR_{ave} [$(ICC/CR_{ave})^2$] improved the fit of the model. There were three final models:

Model	Explanatory variables and covariates	R^2	95% CI	P-value
1	ICC_{ave}*SIZE	0.84	(0.69, 0.87)	<0.0001
2	$(ICC/CR_{ave})^2$	0.84	(0.73, 0.88)	<0.0001
3	ICC_{ave}*CR_{ave}*SIZE	0.93	(0.86, 0.94)	<0.0001

In the simple univariable analysis, ICC_{ave} explained only 56% (35 – 68%) of the variation in gestational age, whereas $(ICC/CR)_{ave}$ explained 75% (61 – 82%) and CR_{ave} explained 86% (78 – 90%). In contrast, a multivariable model (ICC_{ave}, CR_{ave}, SIZE) explained 93% of the variation, but entailed a three-way interaction that was cumbersome to interpret. Similarly, a model that included an interaction between ICC_{ave} and bitch size accounted for 84% of the variation, but required different interpretations for each size category. However, the ICC_{ave}/CR_{ave} ratio squared accounted for 84% of the variation, was independent of bitch size, and had great potential as a user-friendly predictor of gestational age. Data collection and validation of predictive ability are ongoing to establish a user-friendly model to estimate canine gestational age in a practice setting.

Keywords: Canine gestational aging, fetal aging, ultrasound, crown rump

Approaches to canine castration in Nigeria

Ajadi Temitope Ayisat
Department of Veterinary Public Health and Reproduction, Federal University of Agriculture, Abeokuta, Nigeria

Only 1% of dogs in Nigeria are castrated. As part of a larger study into the reasons for this low castration rate, this survey sought to determine the most common methods of castration, the concerns of veterinarians regarding castrations and the level of awareness of veterinarians of alternatives to surgical castration. Questionnaires were distributed to one hundred veterinarians during 2012. The questionnaire established the demography of respondents, recorded their methods of castrations, common complications associated with castration and awareness of alternative castration methods. Descriptive statistics comprising frequency table, cross tabs and chi - square tests were used to analyze data. Statistical analysis was performed with SPSS 19.0 (IBM Corporation, North Castle, NY). Ninety five of 100 (95%) questionnaires were completed. The majority of respondents (52.1 %) had postgraduate qualifications, worked in mixed practices (58.7 %) and had been in practice for ≤10 years (64.2 %). Castrations are often performed on dogs of mixed breeds (42.7 %) and usually for elective reasons (84.3 %). Most (95.6%) castrations are performed surgically. Most (91%) surgical castrations are performed under sedation (xylazine) and local anesthesia (line block with lidocaine). Most respondents administer analgesics (acetaminophen; 54.5 %) or antibiotics (89.9 %) after castration. Some respondents (42.1 %) reported complications after surgical castration, with scrotal swelling (27. 6 %) and scrotal mutilation (11.5 %) listed as the most frequent complications. While the majority of respondents (67.4 %) were aware of alternative castration methods (such as chemical agents and the use of an emasculator), few (9.7 %) have used such methods. The majority of respondents (68.4 %) are willing to adopt non-surgical methods of castration. Level of education, number of years in practice and type of practice had no significant effects (P> 0.05) on the choice of castration method, the frequency of complications or the awareness of alternatives to surgical castration. The results of this survey showed that most veterinarians in Nigeria use the surgical method of castration, and that complications associated with this method occur rather frequently, but are considered relatively insignificant. However, owing to welfare concerns regarding surgical castration in dogs, veterinarians in Nigeria need to be increasingly made aware of non-surgical methods of castration.

Keywords: Castration, veterinarians, dogs, Nigeria

Mucin1 and cytokines mRNA in endometrium of dairy cows with uterine diseases

R. Kasimanickam,[a] V. Kasimanickam,[a] J. Kastelic,[b] A. Tibary[a]
[a]Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, WA; [b]Production Animal Health, College of Veterinary Medicine, University of Calgary, Calgary, AB, Canada

Mucin (MUC) 1 is an inducible innate immune effector and an important component of the first line of defense against bacterial invasion of epithelial surfaces. In human *in vitro* implantation models, MUC1 was lost at the site of embryo attachment in response to blastocyst-derived factors. In addition, there may be an association between MUC1 and infertility, as there is increased endometrial expression in women with a history of failed implantation. The objectives were to evaluate mRNA expression of MUC1 and cytokine genes in the endometrium of cows with various postpartum uterine inflammatory conditions and to determine median days to open (MDO) in diseased cows. Endometrial samples were collected (cytobrush technique) from lactating dairy cows diagnosed with metritis (N=4), endometritis (N=4) and subclinical endometritis (N=4) or no uterine pathology (normal, N=4). These samples were evaluated (quantitative PCR) to determine the mRNA abundances of MUC1, toll-like receptor (TLR) 4, interleukin (IL) β1, IL6, IL8, tumor necrosis factor (TNF)α, insulin-like growth factor (IGF) 1, and IGF binding protein (BP) 2. The mRNA expressions were analyzed by ANOVA using 2-ΔΔCt values to ascertain statistical significance. The difference in MDO between diseased and normal cows was tested by non-parametric (Kruskal-Wallis) test. Prediction of functional gene partners was analyzed using STRING (Version 9.05) for MUC1 in bovine to support the functional aspects of this transcript.

Table. Mean relative mRNA expression* of MUC1 and cytokines in postpartum dairy cows.

Gene	Metritis	Clinical endometritis	Subclinical endometritis	Normal[§]
MUC 1	2.8[b]	2.5[b]	1.3[a]	1.0[a]
TLR4	2.4[b]	2.1[b]	1.4[a]	1.0[a]
IL1β	16.3[c]	13.6[c]	6.4[b]	1.0[a]
IL6	4.6	3.1	2.4	1.0
IL8	17.2[b]	19.4[b]	4.9[a]	1.0[a]
TNFα	13.1[c]	7.3[b]	7.1[b]	1.0[a]
IGF1	3.9[b]	4.5[b]	1.9[a]	1.0[a]
IGFBP2	0.31[b]	0.34[b]	1.4[a]	1.0[a]
MDO	205(191, 220)[d]	147 (134, 157)[c]	132 (126, 138)[b]	116 (100, 123)[a]

*Normalized to endogenous control; [§]Referent; MDO: Median days open; [a-c]Within a row, means without a common superscript differed (P<0.05).

The mRNA expressions were significantly higher for cows with metritis and clinical endometritis compared to normal cows, except for IL6. The mRNA expressions for the target genes were not different for cows with subclinical endometritis, compared to normal cows, except for IL1β and TNFα mRNA (P<0.01). The MDO for cows with uterine disease was greater compared to normal cows (P<0.01). Based on functional protein networks, significant associations were observed between these transcripts.

In conclusion, expression of MUC1 and cytokines were significantly different between the endometrium of normal, fertile versus diseased, subfertile dairy cows. Perhaps these altered gene expressions contribute to endometrial insufficiency and consequently pregnancy wastage.

Keywords: Mucin 1, cytokines, endometrium, uterine disease, dairy cow

Efficacy of short progesterone protocol on previously anestrous does

Kelly L. Chevett, Sandra L. Ayres
Department of Biomedical Sciences, Tufts Cummings School of Veterinary Medicine, North Grafton, MA

The purpose of this project was to compare pregnancy rates in goats bred on the second estrus versus the initial, hormonally induced estrus during the non-breeding season. Twelve Alpine and Saanen dairy goats were used in this study. Does were assigned to either Group 1 or Group 2 for the duration of the experiment, with six does in each group. Blood was drawn throughout the experiment, and serum separated and frozen for later evaluation. Radioimmunoassay was used to measure progesterone levels. All does were induced to cycle with the insertion of a controlled internal drug-releasing device (CIDR) containing progesterone (Day 0). All does received 32 mg of follicle-stimulating hormone (FSH) on Day 2 and 3. On Day 3, CIDR's were removed and all does received 5.0 mg of prostaglandin ($PGF_{2\alpha}$). All does were checked for signs of estrus twice a day after CIDR removal. Does in Group 1 were bred at the first signs of estrus and 12 hours later. Does in Group 2 were not bred on the first cycle, but it was noted if signs of estrus were displayed. On day 5, all does received 50 ug of gonadotropin releasing hormone (GnRH). On day 12, does in Group 2 received 5 mg of $PGF_{2\alpha}$. Does in Group 2 were checked for signs of estrus twice a day on Day 13, 14, and 15 and bred at the first signs of estrus. Does in Group 2 were given a second dose of 50 ug of GnRH on Day 14. Pregnancy rates were determined by progesterone data. One doe had high progesterone levels at day -1 and -2 indicating the presence of a corpus luteum (CL). Progesterone levels on day 1 through 3 rose in all does ranging from 1.7 to 10.6 ng/mL. On the first cycle, all does from Group 1 and 2 came into heat, and all Group 1 does were bred. Two animals in Group 1 produced CLs, with peak progesterone levels ranging from 1.8 to 23.1 ng/mL during their luteal phase, and one animal became pregnant. On the second cycle, two animals came into heat in Group 2 and both were bred. Three does in Group 2 produced CLs with peak progesterone levels ranging from 0.85 to 2.03 ng/mL. None of the goats in Group 2 became pregnant. In conclusion, a short progesterone priming protocol will bring does into heat allowing them to be bred. However percentage of ovulation and CL formation may be low resulting in low pregnancy rates.

Keywords: Progesterone, short protocol, seasonal anestrous

Effect of age on expression of bovine spermatogonia stem cell molecular markers

Mariana Ianello Giassetti, Alexandre Hinkelmann Bruno, Flávia Regina Oliveira de Barros, Robinson André Worst, Giana Carla Pimentel Saurin, Pedro Vale Moreira, Mayra Elena Ortiz Avila Assumpção, José Antonio Visintin

Laboratories of In Vitro Fertilization, Cloning and Animal Transgenesis, Department of Animal Sciences, School of Veterinary Medicine, University of Sao Paulo, Sao Paulo, Brazil

Spermatogonial stem cells (SSCs) have great potential for application in treatments for infertility and animal transgenesis. However, the isolation of bovine SSCs is less efficient than in other species and it is even lower in adult bulls. The present project hypothesis is: expressions of SSC's molecular markers are different between pre-pubertal and adult bovines. The goal of this study was to verify if the expression of PGP 9.5 and integrin alpha six (A6int) are affected by animal age before and after differential plating. In order to perform the experiment, 3 g of parenchyma of testicles from pre-pubertal animals (5 months old, n=10) and bulls (3-4 years old, n=10) were minced and digested with collagenase (1 mg/ml) for 30 min at 37°C followed by trypsin (2.5 mg/ml) for 5 min at 37°C. Cells were plated ($3x10^6$ viable cells/ plate) in 100 mm culture dishes covered with BSA (0.5 mg/ml). Cells were cultured for 18 hours in high humidity atmosphere with 5% of CO_2 at 37°C. Approximately $10x10^6$ viable cells from the supernatant were fixed with cold ethanol 70% and incubated with antibody anti A6Int labeled with Alexa Fluor 488 (BioLegend, San Diego, CA) or antibody anti PGP 9.5 (Abcam, Cambridge,MA) for 30 min at 4°C. Samples previously incubated with anti PGP 9.5 antibody were incubated with a second antibody labeled with FITC for 40 min at 4°C. The percentage of cells labeled by the green fluorescence was determined by flow cytometry (Atunne, Applied Biosystems, Foster City, CA). The non-specific fluorescence was subtracted for each sample. Presence of positive cells for A6Int and PGP 9.5 was evaluated by Tukey's Studentized Range (SAS, Cary, NC) and the effect of each testicle in this percentage by T-test (SAS). No effect of age was observed on the percentage of A6int and PGP 9.5 positive cells after the purification method. However, an age effect was observed on expression of PGP 9.5 in pre-pubertal animals before the differential plating and this expression was higher (P=0.0489). In conclusion, the molecular markers to SSCs were expressed at the same proportion in cells purified from pre-pubertal and adult testis. Differential plating does not increase the percentage of SSCs as was expected.

Keywords: Spermatogonia, cytometry, PGP9.5, integrin alpha six, bovine

Detection of genes encoding multidrug resistance and biofilm virulence factor of uterine pathogenic bacteria in postpartum dairy cows

Katilyn Hanson, Vanmathy Kasimanickam, Ram Kasimanickam
Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, WA

Reckless use of antibiotics and/or development of biofilm are the rationale for the development of multidrug resistance (MDR) of pathogenic bacteria. The objective was to detect MDR genes in *Trueperella pyogenes* (16SrRNA, *intI 1*, *intI 2*, and cassette genes) and to detect biofilm virulence factor (VF) genes (Agn43aCFT073 and Agn43bCFT073) in *Escherichia coli* isolated from the uterus of postpartum dairy cows. Uterine secretions from all parity postpartum Holstein cows (N=40) were collected using cytobrush technique following a sterile procedure as part of diagnosing cows with uterine inflammatory conditions. The cytobrush brush was stored in specimen collector (Diagnostic Systems, Sparks, MD), placed in a cooler with ice and transported to the laboratory within 2 h. The pathogens were isolated strictly following methods described. Initial identification was based on colony morphology and biochemical characteristics. Pure cultures were isolated. To further identify and classify the single species, and to determine the presence of MDR and VF genes, the genes fragments were amplified using the respective primers (NCBI) by either singleplex or multiplex polymerase chain reaction (PCR) protocol. Further, PCR products were run on agarose gel and viewed after ethidium bromide staining to make certain a single amplicon for a set of primer (Figure).

Figure. Photograph of the ethidium bromide-stained electrophoresis gel, with amplicons of the expected sizes

T. pyogenes isolates were positive for the presence of *intI 1* gene and gene cassettes. Five cows were *intI 1* positive. Of those five, four cows were gene cassette positive. No cows were *intI 2* positive. The 1048 and1608- bp amplicon revealed presence of *aadA 5* and *aadA 24-ORF1* gene, respectively. The *aadA 5* indicated resistance to sulfadiazine, bacitracin, florfenicol and ceftiofur. The *aadA 24-ORF1* indicated resistance to sulfadiazine, bacitracin, penicillin, clindamycin and erythromycin. The VF genes, Agn43a and b were present in *E. coli* isolates from persistently infected cows.

In conclusion, detection of *intI 1* and gene cassettes can be associated with integron-cassette mediated multidrug resistance in *T. pyogenes* isolates. Presence of VF genes indicated the formation of biofilm in the uterus of persistently infected cows possibly contributing to multidrug resistance in *E. coli* isolates.

Keywords: Dairy cows, postpartum uterus, bacteria, multidrug resistance, virulence factor

Mucin1 and cytokines mRNA in endometrium of dairy cows with uterine diseases

R. Kasimanickam,[a] V. Kasimanickam,[a] J. Kastelic,[b] A. Tibary[a]
[a]Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, WA; [b]Production Animal Health, College of Veterinary Medicine, University of Calgary, Calgary, AB, Canada

Mucin (MUC) 1 is an inducible innate immune effector and an important component of the first line of defense against bacterial invasion of epithelial surfaces. In human *in vitro* implantation models, MUC1 was lost at the site of embryo attachment in response to blastocyst-derived factors. In addition, there may be an association between MUC1 and infertility, as there is increased endometrial expression in women with a history of failed implantation. The objectives were to evaluate mRNA expression of MUC1 and cytokine genes in the endometrium of cows with various postpartum uterine inflammatory conditions and to determine median days to open (MDO) in diseased cows. Endometrial samples were collected (cytobrush technique) from lactating dairy cows diagnosed with metritis (N=4), endometritis (N=4) and subclinical endometritis (N=4) or no uterine pathology (normal, N=4). These samples were evaluated (quantitative PCR) to determine the mRNA abundances of MUC1, toll-like receptor (TLR) 4, interleukin (IL) β1, IL6, IL8, tumor necrosis factor (TNF)α, insulin-like growth factor (IGF) 1, and IGF binding protein (BP) 2. The mRNA expressions were analyzed by ANOVA using 2-ΔΔCt values to ascertain statistical significance. The difference in MDO between diseased and normal cows was tested by non-parametric (Kruskal-Wallis) test. Prediction of functional gene partners was analyzed using STRING (Version 9.05) for MUC1 in bovine to support the functional aspects of this transcript.

Table. Mean relative mRNA expression* of MUC1 and cytokines in postpartum dairy cows.

Gene	Metritis	Clinical endometritis	Subclinical endometritis	Normal[§]
MUC 1	2.8[b]	2.5[b]	1.3[a]	1.0[a]
TLR4	2.4[b]	2.1[b]	1.4[a]	1.0[a]
IL1β	16.3[c]	13.6[c]	6.4[b]	1.0[a]
IL6	4.6	3.1	2.4	1.0
IL8	17.2[b]	19.4[b]	4.9[a]	1.0[a]
TNFα	13.1[c]	7.3[b]	7.1[b]	1.0[a]
IGF1	3.9[b]	4.5[b]	1.9[a]	1.0[a]
IGFBP2	0.31[b]	0.34[b]	1.4[a]	1.0[a]
MDO	205(191, 220)[d]	147 (134, 157)[c]	132 (126, 138)[b]	116 (100, 123)[a]

*Normalized to endogenous control; [§]Referent; MDO: Median days open; [a-c]Within a row, means without a common superscript differed (P<0.05).

The mRNA expressions were significantly higher for cows with metritis and clinical endometritis compared to normal cows, except for IL6. The mRNA expressions for the target genes were not different for cows with subclinical endometritis, compared to normal cows, except for IL1β and TNFα mRNA (P<0.01). The MDO for cows with uterine disease was greater compared to normal cows (P<0.01). Based on functional protein networks, significant associations were observed between these transcripts.

In conclusion, expression of MUC1 and cytokines were significantly different between the endometrium of normal, fertile versus diseased, subfertile dairy cows. Perhaps these altered gene expressions contribute to endometrial insufficiency and consequently pregnancy wastage.

Keywords: Mucin 1, cytokines, endometrium, uterine disease, dairy cow

Effect of GnRH at CIDR insertion on Day 0 and number of PGF2a doses at CIDR removal on Day 5 on AI pregnancy rate in heifers synchronized with 5-d CO-Synch + CIDR program

R. Kasimanickam,[a] G. Schuenemann,[b] B. Whitlock,[c] D. Moore,[a] J. Hall,[d] W. Whittier[e]
[a]Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, WA; [b]Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University, Columbus, OH; [c]Department of Large Animal Clinical Sciences, College of Veterinary Medicine, University of Tennessee, Knoxville, TN; [d]Nancy M. Cummings Research Extension and Education Center, University of Idaho, Carmen, ID; [e]Department of Large Animal Clinical Sciences, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA

The objective was to determine the effect of gonadotropin releasing hormone (GnRH) at controlled internal drug release insert (CIDR) insertion on Day 0 and number of prostaglandin F2α (PGF) doses at CIDR removal on Day 5 on artificial insemination pregnancy rate (AI-PR) in a 5-d CO-Synch + CIDR program in heifers. Angus cross beef heifers (N = 1018) at eight locations and Holstein dairy heifers (N=1137) at 15 locations were included in this study. On Day 0, heifers were given a body condition score (BCS), and received a CIDR (Eazi-Breed™ CIDR® Cattle Insert; Pfizer Animal Health, New York, NY) insert. A subset of heifers (N=318) were bled for serum progesterone estimation and/or scanned for presence of corpus luteum (CL) on ovaries by ultrasound. Within farms, heifers were randomly divided into two groups: GnRH group received 100 μg of GnRH im (Factrel®, Pfizer Animal Health) and No-GnRH group received no treatment at the time of CIDR insertion. On Day 5, all heifers received 25 mg of PGF (Lutalyse® sterile solution; Pfizer Animal Health) at the time of CIDR insert removal. The GnRH and No-GnRH groups were further divided into 1PGF and 2PGF groups. The heifers in 2PGF group received a second dose of PGF 6 h after the first dose. Beef heifers were inseminated at 56 h and dairy heifers at 72 h from CIDR removal and received 100 μg of GnRH concurrently. Pregnancy was determined 35 and/or 70 days after AI. Analysis was performed separately for beef and dairy heifers using Proc Glimmix of SAS (version 9.3) to determine differences in AI-PR. Locations, AI sires and AI technicians were offered as random effects. The results were presented in the table below.

Treatment	Beef heifers[§]		Dairy heifers[♣]	
	N	AI-PR	N	AI-PR
No-GnRH+PGF+GnRH	290	50.3[a]	291	51.2[a]
No-GnRH+2PGF+GnRH	237	50.2[a]	283	51.9[a]
GnRH+PGF+GnRH	263	59.7[b]	284	53.9[a]
GnRH+2PGF+GnRH	228	58.3[ab]	279	54.5[a]

[ab]Different superscripts within column were different (P<0.05); [§]Beef heifer AI-PR varied among locations from 50 to 62.4%; [♣]Accounted for BCS differences in AI-PR (P<0.05). Dairy heifer AI-PR varied among locations from 48.3 to 75.0%.

Numerically higher AI-PR were observed in beef and dairy heifers that exhibited high progesterone concentrations at the time of CIDR insertion (>1 ng/mL, with a CL). In addition, numerically higher AI-PR were also observed in heifers receiving CIDR + GnRH with both high and low progesterone concentration (< 1ng/mL) initially compared to heifers receiving a CIDR only with low progesterone.

In conclusion, GnRH administration at the time of CIDR insertion is advantageous in beef heifers to synchronize follicular wave emergence and to improve AI-PR in the 5-d CIDR+CO-Synch synchronization protocol. In addition, heifers in both no GnRH and GnRH groups that received either one or two PGF doses resulted in similar AI-PR in this study.

Keywords: Heifers, five-day CIDR, GnRH, two PGF2α, pregnancy rate

Kisspeptin receptor agonist (FTM080) increased plasma concentrations of luteinizing hormone in anestrous ewes

Brian K. Whitlock,[a] Kelly A. Chameroy,[a] Rebecca R. Payton,[b] Shinya Oishi,[c] Nobutaka Fujii,[c] Joseph A. Daniel[d]

[a]Department of Large Animal Clinical Sciences, College of Veterinary Medicine, The University of Tennessee, Knoxville, TN; [b]Department of Animal Science, The University of Tennessee, Knoxville, TN; [c]Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan; [d]Department of Animal Science, Berry College, Mt. Berry, GA

Kisspeptin (KP) and the KP receptor (Kiss1r) are integral to central regulation of the gonadotropic-axis. The demonstration that intravenous infusion of KP can stimulate gonadotropin secretion and ovulation in seasonally anestrous female sheep offers a means of manipulating the reproductive axis. However, KP may be of limited clinical use because of the short circulating half-life. Kiss1r agonists with increased half-life and similar efficacy to KP *in vitro* potentially may provide beneficial applications in breeding management of many species. However, many of these agonists have not been tested *in vivo*. This study was designed to test and compare the effects of a Kiss1r agonist (FTM080) and KP on luteinizing hormone (LH) *in vivo*. Sheep (n = 4 per treatment) were treated with KP (500 pmol/kg BW), one of three dosages of FTM080 (500, 2500, or 5000 pmol/kg BW), or sterile water (VEH) in a 2-ml bolus via jugular catheter. Serial blood samples were collected every 15 minutes before (1 hr) and after (4 hr) treatment. Plasma concentrations of LH were tested for effect of treatment, time, and treatment by time interaction using ANOVA procedures for repeated measures. Area under the LH curve was tested for effect of treatment, period (pre- or post-treatment), and treatment by period interaction using ANOVA procedures for repeated measures. Means separation was performed using Student's T test when appropriate. Plasma LH concentrations following treatment with KP were greater than ($P < 0.05$) VEH and low-dose FTM080 (500 pmol/kg) through the 45-min sample and middle-dose FTM080 (2500 pmol/kg) at 30 min. Plasma LH concentrations following high-dose FTM080 (5000 pmol/kg) was greater than ($P < 0.05$) VEH through the 30-min sample and low-dose FTM080 (500 pmol/kg) at the 15-min samples. The area under the curve (AUC) of LH in the period from 0 to 60 min (post-treatment) following KP was greater than ($P < 0.05$) all other treatments and the post-treatment AUC of LH following high-dose FTM080 (5000 pmol/kg BW) was greater than ($P < 0.05$) all treatments except KP. The AUC of LH in the post-treatment period was greater than ($P < 0.05$) the AUC of LH in the pre-treatment period (-60 to 0 min) for the low-dose FTM080 (500 pmol/kg) and middle-dose FTM080 (2500 pmol/kg). In conclusion, these data provide evidence to suggest that FTM080 stimulates the gonadotropic axis of sheep *in vivo*. Any increased half-life and comparable efficacy of FTM080 to KP *in vitro* does not appear to translate to *in vivo*.

Keywords: Kisspeptin, agonist, luteinizing hormone, sheep

Aberrant follicular dynamics in dairy cows due to subluteal levels of progesterone after incomplete luteolysis or CIDR application during and after superovulation

J-P Pelletier, R.C. Lefebvre, A.E. Stock
Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, QC, Canada

Many cows do not return to normal estrous cyclicity after embryo collection. We therefore followed three Holstein dairy cows in a research study on superovulation and embryo collection by ultrasound and blood sampling to measure peripheral progesterone twice weekly. In the first case (Lola), luteal regression was induced by two injections of prostaglandin on Day 7 and 8 and one CIDR (CIDR® 1380, Pfizer Animal Health, Pfizer Canada Ltd, Kirkland QC, Canada) was inserted at the time of first prostaglandin injection. The CIDR alone created circulating levels of progesterone (0.7 to 1.3 ng /ml). The superovulation protocol starting on Day 11 failed to result in multiple follicle formation and ovulation. Instead, a single huge persistent follicle developed, ovulated after CIDR withdrawal and formed into an abnormally large corpus luteum (CL) producing 9.0 ng/ml of progesterone. A second cow (Martha) was followed after embryo collection. This cow demonstrated progesterone levels that did not decline completely, but remained detectable (>0.2 ng/ml) although she had received two injections of prostaglandin at two and four days after embryo collection. An injection of gonadotropin releasing hormone induced the ovulation of a persistent follicle, which formed a CL with cavity secreting levels of only 1.0 ng/ml of progesterone. Induced luteolysis of this CL and the insertion of two CIDRs resulted in 0.9 to 1.6 ng/ml of circulating progesterone which was accompanied by newly forming persistent follicles. In this specific cow, only the insertion of three CIDRs and the injection of 100 mg of progesterone twice daily raised circulating progesterone levels up to 4.2 ng/ml. The withdrawal of CIDRs and the discontinuation of progesterone injections after one week resulted in ovulation and normal CL formation and pregnancy thereafter. In the third dairy cow (Monica) a follicle that persisted until the day of embryo collection developed into a follicular cyst once the two injections of prostaglandin were administered after the embryo collection, while the CLs slowly regressed. A treatment was put in place with the application of two CIDRs, which achieved circulating levels of progesterone of 3.3 to 3.5 ng/ml. Two new cysts appeared during this treatment. The two CIDRs were replaced with new CIDRs and 100 mg of progesterone twice daily was added, achieving a plasma concentration of over 6.0 ng/ml. The treatment was followed by a normal ovulation and CL formation. From these observations we conclude that incomplete luteolysis resulting in residual circulating levels of progesterone could cause the development of persistent follicles, anovulation and poor CL formation. Depending on the animal, the intravaginal insertion of one or even two CIDRs may not be sufficient to increase peripheral progesterone to normal luteal levels, and may result in abnormal cyclic function. Progesterone testing combined with ultrasonography may be a valuable tool to foresee and diagnose aberrant follicular dynamics in individual dairy cows.

Keywords: Abnormal follicle development, progesterone, CIDR, prostaglandin

Inflammatory endometrial response to two different uterine lavage solutions in donor mares

D.F. Mogollon,[a] M.O. Benoit-Biancamano,[b] I. Raggio,[a] P. Poitras,[a] D. Vaillancourt,[a] A.E. Stock,[a] R.C. Lefebvre[a]

[a]Department of Clinical Sciences and [b]Pathology, Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, Québec, Canada

Embryo transfer has become a commonly applied technique in equine reproduction. Although it is expected that embryo lavage solutions elicit an acute, but transient inflammatory uterine response in the mare, we hypthesized that different solutions may have a different inflammatory effect. The objective was to compare the acute inflammatory response of healthy mares after embryo collection using two commonly used lavage solutions (A= lactated Ringer's; B= EquiPRO®; Minitube of America, Ingersoll, ON, Canada). Mares (n=21) with normal reproductive tracts confirmed by transrectal, vaginal, ultrasonographic and cytological examinations were induced in estrus and inseminated with fresh, extended semen (500 million motile sperm) from the same stallion as soon as the ovulatory follicle had reached a diameter of 35 mm and the uterus demonstrated clear edema. Ovulation was induced (2500 IU human chorionic gonadotropin IV) and monitored by ultrasonography every 24 hours. At day 7.0 after ovulation, mares were randomly assigned for embryo collection with one of the two lavage solutions in a cross-over double blind study. A normal estrous cycle was allowed between the two collections. Uterine cytology, ultrasonography and transrectal palpation were performed at T0 (immediately before the embryo collection), 24h and 48h after the embryo collection. For the statistical analysis, the data were transformed (arcsinus of square root). The ratios of neutrophils (number of PMN/total number of cells at 400X) at T0 were significantly smaller compared to the two other sampling times for both solutions (A: T24 p=0.0001, T48 p=0.0001; B: T24 p=0.005, T48 p=<0.004) without significant differences between solutions (T0 p=0.99, T24 p=0.24, T48 p=0.11). Although repeated embryo collection and quality of the embryos were not affected by the use of both solutions (unpublished data) it is not known if the use of either solution has long term effects on fertility of donor mares.

Keywords: Endometritis, uterine lavage, neutrophils, mares.

The distribution of carrier status of *T. equigenitalis* in stallions and exposed mares in South Africa

C.E. May, M.L. Schulman, B. Keys, A. Guthrie
University of Pretoria, Gauteng, South Africa

Taylorella equigenitalis, the causative agent associated with contagious equine metritis (CEM), colonizes the external genitalia of carrier stallions and a variable proportion of exposed mares. Following a recent CEM outbreak in South Africa, surveillance of the national herd was instituted to determine the prevalence of *T. equigenitalis* carriers.

We hypothesised a higher proportion of stallions than mares in the exposed population would be identified as carriers of *T. equigenitalis*, associated with reported differences in persistence of the carrier status. This study aimed to determine both the overall prevalence of *T. equigenitalis* and its distribution between carrier stallions and mares.

Between April 2011 and end February 2013, a total of 2265 males and 68 exposed mares of all ages and 65 different breeds were screened for *T. equigenitalis* via swabs obtained from the external genitalia in males and mares, and the endometrium of non-pregnant mares for qPCR analysis. A critical value ($C_T < 40$) was the threshold for detection (Table). All suspect positive carriers were confirmed on bacteriology according to the World Organization for Animal Health (OIE) requirements. The detection frequency for *T. equigenitalis* between positive males and mares was determined.

Horses sampled	qPCR	
	Positive ($C_T < 40$)	Negative
Males (n=2265)	36	2229
Mares (n=68)	3	65
Total (n=2333)	39	2294

Table: Distribution of positive & negative horses based on qPCR testing in the sampled population

An overall prevalence of 1.67%, with 36/2265(1.59%) of males and 3/68 (4.41%) of exposed mares among sampled animals was observed. These findings failed to support the hypothesis and a previous report of an increased frequency of detection from stallions during outbreak surveillance.[1] This is probably associated with the sample population bias, supporting the inclusion of exposed mares during targeted epidemiologic surveillance of stallions.

Keywords: Carrier, *T.equigenitalis*, qPCR, surveillance, genital swab, horse

Reference

1. Erdman MA, Creekmore LH, Fox PE, et al: Diagnostic and epidemiologic analysis of the 2008–2010 investigation of a multi-year outbreak of contagious equine metritis in the United States. Prev Vet Med 2011;101:219-228.

The distribution of *Taylorella equigenitalis* on the external genitalia of carrier stallions in South Africa

C.E. May, M.L. Schulman, B. Keys, A.J. Guthrie
University of Pretoria, Gauteng, South Africa

The bacterium *Taylorella equigenitalis,* associated with contagious equine metritis (CEM), colonizes predilection sites within the external genitalia of male horses. This colonization is reportedly variable with respect to both anatomic site and concentration, necessitating sequential and multi-site testing for accurate detection in carrier animals. We hypothesised a single predominant predilection site for *T. equigenitalis* in carrier stallions. The study aimed to determine both the organisms' frequency and load at the three reported predilection sites.

Following a recent CEM outbreak in South Africa, 2661 breeding stallions were screened in the course of a nation-wide surveillance for *T. equigenitalis* between April 2011 and January 2013. All stallions were sampled by obtaining two sets ($\geq$ 7 d apart) of three swabs obtained from: urethra (swab 1); urethral fossa including sinus (swab 2); and lamina interna (swab 3). Swabs were analyzed using a duplex qPCR.[1] A total of 36 suspect positive stallions identified on qPCR were all confirmed via bacteriology. The critical threshold value (Ct) was determined by site and a Ct < 40 was the threshold for detection. These molecular data were retrospectively organized into three categories describing bacterial load: 30-35 (low); 21-29 (moderate); and $\leq$ 20 (strong). The Ct values at different sites were compared using analysis of variance (ANOVA) on ranks using Dunn's method for multiple comparisons, with statistical significance set at $p < 0.05$.

Bacterial load	Predilection site			Total
	urethra (swab 1)	urethral fossa (swab 2)	lamina interna (swab 3)	
Ct $\leq$ 20	0	4	0	4
Ct 21- 29	13	11	10	34
Ct 30-39	8	6	9	23
Negative	3	2	5	10
Ct value (ave.)	29.56	25.98*	32.12*	

Table. The distribution of *T. equigenitalis* by molecular detection frequency and level (sensitivity) after swabbing predilection sites in carrier stallions (n=24) for qPCR analysis. *Significantly different ($p < 0.05$)

In 12 of the positive stallions, only pooled Ct samples were available, and were not included in the study. In the 24 remaining positive stallions, the bacterial load in the urethral fossa was significantly higher than the lamina interna. These findings support correct sampling technique and site of sampling in the diagnosis of *T. equigenitalis* in carrier stallions.

Keywords: Stallion, *T. equigenitalis,* PCR, carrier, genital swab, horse

Reference

1. Wakeley PR, Errington J, Hannon S, et al: Development of a real time PCR for the detection of *Taylorella equigenitalis* directly from genital swabs and discrimination from *Taylorella asinigenitalis*. Vet Microbiol 2006;118:247-254.

Effect of LDL on stallion sperm motility after cryopreservation

R. Susan Rodgers, Heath King, Richard Hopper
Department of Pathobiology and Population Medicine, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS

Cryopreservation and subsequent thawing places several stressors on cells, the most detrimental include ice crystal formation, osmotic changes, temperature shock and physical manipulation. Spermatozoa of different species tolerate the cryopreservation process differently. For example, stallion spematozoa do not tolerate the freezing process as well as bull or human spermatozoa. Current methods to improve freezability include altering the freezing media, freezing process and thawing protocol. Egg yolk improves post-thaw quality of spermatozoa, and it has been suggested that the cryoprotectant properties of egg yolk are found in the low density fraction which is primarily composed of low density lipoprotein (LDL). Low density lipoprotein has been shown to improve post-thaw quality of dog, bull, boar and ram spermatozoa. Simply adding clarified egg yolk plasma has been shown to improve stallion freezing quality.[1]

Thus, the goal of this project was to determine if the addition of LDL to freezing media at different concentrations could improve the post-thaw motility of stallion spermatozoa compared to that of semen frozen with clarified egg yolk plasma. Two extenders were used: INRA 96 (IMV, Maple Grove, MN) and a modified Kenney extender. Four stallions of three breeds ranging from 4-18 years of age were each collected eight times for a total of 32 collections. Each collection was divided into 10 aliquots and cryopreserved using standard methodology in INRA 96 and a modified Kenney extender with clarified egg yolk or 2,4,6,8% LDL added. Egg yolk was clarified by centrifugation and LDL was extracted from fresh chicken eggs by a combination of centrifugation and induced salt formation.[2] Post-thaw motility of the samples was assessed by computer assisted sperm analysis (CASA) and the data were analyzed using the SAS mixed procedure method. Post-thaw progressive motility did not differ significantly ($P > 0.1$) between extenders containing LDL and clarified egg yolk. A significant reduction ($P < 0.03$) in total motility post-thaw was observed in extenders containing 6 and 8% LDL from clarified egg yolk. When semen extenders were analyzed separate from each other the addition of LDL to the INRA extender did not ($P > 0.1$) significantly affect total motility. However, when the modified Kenney extender was used with higher levels of LDL, it significantly reduced ($P < 0.02$) total motility post-thaw compared to clarified egg yolk. It is clear that higher levels of LDL were not beneficial to post-thaw motility in this study.

Keywords: Low density lipoprotein, stallion, cryopreservation, CASA

References

1. Pillet E, Duchamp G, Batellier F, et al: Egg yolk plasma can replace egg yolk in stallion freezing extenders. Theriogenology 2011;75:105-114.
2. Moussa M, Martinet V, Trimeche A, et al: Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. Theriogenology 2002;57:1695-1706.

Analysis of two formulations of ceftiofur in the seminal plasma of stallions

Aime K. Johnson,[a] Robyn R. Wilborn,[a] Erin Aufox,[a] Jacob A. Johnson,[a] Nathan Voris[b]
[a]Department of Clinical Sciences, College of Veterinary Medicine, Auburn University, Auburn, AL;
[b]Equine Technical Services, Pfizer Animal Health, New York, NY

Treating infections within the reproductive tract of stallions presents many challenges. Little is known regarding which systemic antibiotics successfully enter the reproductive tract or the dosage needed to achieve effective concentrations within the target tissues. In this experiment, six stallions received two formulations of injectable ceftiofur, either ceftiofur sodium (CS; Naxcel®, Pfizer Animal Health, New York, NY) or ceftiofur crystalline-free acid (CCFA; Excede®, Pfizer Animal Health) in a randomized cross-over design utilizing a 14 d washout period between the two formulations. Both formulations were administered at the manufacturer's recommended dose for a 10 d treatment period (CS: 2.2 mg/kg, i.m., q 24 h; CCFA: 6.6 mg/kg, i.m., q 96 h). The ceftiofur concentration in seminal plasma and blood plasma were determined by HPLC-MS/MS on days one, three, five, seven, nine, and ten. Total sperm number, total and progressive motility, velocity, and morphology were evaluated both prior to and throughout the treatment period. We hypothesized that concentrations of both forms of ceftiofur would be detectable in the seminal plasma of stallions. Results are reported as mean ± SD.

Over the 10-day study period, semen quality either remained constant or improved for all stallions. The mean blood plasma remained above the Food and Drug Administration's Center for Veterinary Medicine clinical breakpoint for ceftiofur activity against *Streptococcus equi* subsp. *zooepidemicus* (200 ng/mL) for the entire study period with both formulations (CS: 469.1 ng/mL ± 122.6 and CCFA: 709.6 ng/mL ± 430.3). The mean seminal plasma concentrations of ceftiofur in stallions receiving CS and CCFA were 175.0 ng/mL ± 154.2 and 87.9 ng/mL ± 57.9, respectively. The mean seminal plasma concentration of ceftiofur in stallions receiving CCFA, was highest on days three and seven (130.9 ng/mL ± 74.5 and 104.2 ng/mL ± 78.1, respectively), while the highest blood plasma concentrations occurred on days one and five (1230.7 ng/mL ± 340.4 and 880.8 mg/mL ± 642.8, respectively). The mean seminal plasma concentration of ceftiofur in stallions receiving CS was highest on days three and five (234.3 ng/mL ± 265.7 and 274.3 ng/mL ± 200.3), while the blood plasma concentrations on those days reached 488.2 ng/mL ± 81.9 and 500.8 ng/mL ± 139.6, respectively.

Both formulations of ceftiofur administered at the recommended dose resulted in detectable levels in the seminal plasma but failed to consistently achieve concentrations in the seminal plasma exceeding the FDA's clinical breakpoint.

Keywords: Ceftiofur; seminal plasma; stallion; antibiotic

Ultrasonographic characterization of the accessory sex glands in normal geldings

M.R. Schnobrich, R.O. Turner, J. Slack
University of Pennsylvania School of Veterinary Medicine, New Bolton Center, Kennett Square, PA

Transrectal ultrasonographic examination of the internal genital tract is an important tool for examination of reproductive tract pathologies in stallions. The normal ultrasonographic appearances and sizes of the stallion accessory sex glands have been described and are often used as reference points to aid in the diagnosis of pathologies such as seminal vesiculitis and ampullary blockage.[1] In spite of the fact that pathologies of the accessory sex glands have been reported in geldings,[2] an ultrasonographic description of the accessory sex glands of normal geldings has not been reported to our knowledge. A detailed understanding of the ultrasonographic character of the normal internal reproductive tract of the gelding would be a valuable clinical tool when examining geldings with suspected pathologies. The objective of this study was to characterize the ultrasonographic appearance of the accessory sex glands of geldings and to establish normal ranges for accessory sex gland sizes. The hypothesis is that the accessory sex glands can be reliably imaged in normal geldings and that an established range of measurements for normal glands can be used as a tool to aid diagnosis of suspected pathology.

Keywords: Accessory sex glands, ultrasound, gelding

Methods

Twelve mature, clinically normal, light horse geldings, two to 25 years of age and with histories of normal castrations were the subjects of this study. Transrectal ultrasonographic evaluation of the internal urogenital tract was performed as previously described for stallions and with a goal of obtaining 24 separate measurements of the various components of the internal urogenital tract for each animal.[1,3] Images were obtained using both a 7.5 MHz linear-array transducer and a 6.0-10 MHz microconvex linear-array transducer. Descriptive statistics were compiled for each measurement.

Results

A full complement of accessory glands was identified in all animals and 281 of the 288 (97.6%) possible measurements were successfully obtained. Means, ranges and standard deviations were calculated for each measurement. In general, accessory sex glands were similar in appearance, although generally smaller, than those described for stallions. Intra-luminal fluid was frequently present in the seminal vesicles (nine out of 12 geldings).

Discussion

We conclude that the accessory sex glands can be reliably imaged in normal geldings. We have established normal ranges for a variety of dimensions of the accessory glands, based on this sample population of 12 normal animals. We have successfully applied these values to the evaluation of geldings presenting to our hospital for clinical signs associated with the internal urogenital tract, thus demonstrating the utility of these data.

References

1. Pozor MA, McDonnell SM: Ultrasonographic measurements of accessory sex glands, ampullae, and urethra of normal stallions of various size types. Theriogenology 2002;58:1425-1433.
2. Knobbe M, Levine D, Habecker P, et al: Prostatic masses in geldings. J Equine Vet Sci 2012;32:628-633.
3. Diaz OS, Smith G, Reef VB: Ultrasonographic appearance of the lower urinary tract in fifteen normal horses. Vet Radiol Ultrasound 2012;48:560-564.

Effect of L-arginine supplementation on follicular hemodynamics in the mare – preliminary findings

H.L. Resende,[a] H.S. Canesin,[a] C. Ramires-Neto,[a] F.L.V. Pinaffi,[b] L.A. Silva,[b] M.A. Alvarenga[a]
[a]Department of Animal Reproduction; School of Veterinary Medicine and Animal Science (FMVZ), UNESP, Sao Paulo State University; Botucatu SP,Brazil; [b]Dr. O.J. Ginther Theriogenology Laboratory; Department of Veterinary Medicine; School of Animal Science (FZEA), USP, University of Sao Paulo, Sao Paulo, Brazil

Vascularization of the pre-ovulatory follicle has been positively correlated with pregnancy rates in mares,[1] and administration of the amino acid L-arginine (Arg) caused an increase in uterine blood flow and acceleration of postpartum uterine involution in mares.[2] The present study aimed to evaluate vascular perfusion of the ovary and follicles at the time of induction of ovulation in mares supplemented with Arg. Sixteen mares between three and eight years of age were randomly divided in two groups: mares supplemented with (n=8) or without (n=8) 100 g Arg (Ajinomoto Amino Science LLC, Raleigh, NC) in 3 kg of feed twice daily beginning 8 d after ovulation until the following induced ovulation. Transrectal B-mode ultrasound examination was performed to monitor follicular dynamics. Upon detection of a follicle $\geq$ 35 mm in diameter, spectral and color Doppler ultrasonography were used to assess ovarian and follicular vascular perfusion before and after ovulation induction. Follicular vascularization was subjectively estimated in real time Doppler mode and was graded with a score ranging from 0 to 100%, based on the percentage of follicular wall with evidence of vascularization. The vascular perfusion of the ovarian artery ipsilateral to the pre-ovulatory follicle was evaluated by the resistance (RI) and pulsatility index (PI) using spectral Doppler mode. These indices represent inverse relationships with vascular perfusion of the target tissue. To obtain these indices, the cursor was positioned in an artery of the ovarian pedicle and three identical spectral graphics of subsequent cardiac cycles were generated to obtain the RI and PI values. A set of measurements were obtained before, and at 24 and 48 h after, administration of deslorelin acetate (1 mg, IM). The distribution of the variable responses were analyzed by the Shapiro-Wilk test, and differences between groups were evaluated by LSD tests and non-paired t-tests (SAS 9.2, SAS Institute, Inc., Cary, NC). The level of statistical significance was set as 0.05. There was no difference in PI ($P = 0.42$), RI ($P = 0.60$) and subjective follicular vascularization ($P = 0.65$) between mares supplemented with Arg and control mares. In young mares, Arg supplementation did not elevate vascular perfusion of the pre-ovulatory follicle. Several factors are involved in the regulation of ovarian hemodynamics, and further studies are warranted.

Keywords: Doppler; follicular hemodynamics; l-arginine; mare

References

1. Silva LA, Gastal EL, Gastal MO, et al: Relationship between vascularity of the preovulatory follicle and establishment pregnancy in mares. Anim Reprod 2006;3:339-346.
2. Mortensen CJ, Kelley DE, Warren LK: Supplemental l-arginine shortens gestation length and increases mare uterine blood flow before and after parturition. J Equine Vet Sci 2011;31:514-520.

Temporary aspermia following equine viral arteritis (EVA) vaccination in a stallion

Michelle Kutzler,[a] Sandra Lloyd[b]
[a]Department of Animal and Rangeland Sciences, Oregon State University, Corvallis, OR; [b]Banfield Pet Hospital, Reno, NV

Significance

The following case demonstrates an unlikely sequel that developed following EVA vaccination in a stallion.

Case report

Aspermia with bilaterally enlarged ampullae was diagnosed in a 17-year-old Appaloosa stallion by the referring veterinarian during a prebreeding season fertility evaluation. Despite repeated collections with rectal massage of the ampullae and treatment with oxytocin and prostaglandin $F_{2\alpha}$, a normal ejaculate could not be obtained. All mares bred by this stallion in the previous breeding season became pregnant. The owner was concerned that the cause of the stallion's aspermia was the result of EVA vaccination (Arvac®; Zoetis; Madison, NJ) that was administered eight weeks previously. Three weeks following diagnosis of aspermia from the referring veterinarian, the stallion was presented to the Oregon State University Veterinary Teaching Hospital. On presentation, the testes had a turgid consistency with a scrotal width of 10 cm. Transrectal palpation and ultrasonography revealed bilaterally enlarged ampullae. The stallion had normal libido when presented to an estrous mare and mounted the phantom and ejaculated on the first attempt. Examination of the ejaculate revealed few sperm (<1 million/mL) with concurrent low seminal alkaline phosphatase (8 U/L), consistent with a lack of caudal epididymal secretions. Semen bacterial culture yielded minimal growth of α-hemolytic *Streptococcus* sp. (1+) and *Corynebacterium* sp. (2+), consistent with normal flora. Virus isolation and polymerase chain reaction of the ejaculate were negative for the equine arteritis virus. Urethroscopy was performed, revealing an inflamed soft tissue mass on the seminal colliculus. The significance of this mass was unknown as the openings of the ampullae and the seminal vesicles appeared to be normal and unobstructed. Testosterone, luteinizing hormone and follicle stimulating hormone were measured by radioimmunoassay and were within normal limits.

Follow up

The stallion was re-evaluated by the referring veterinarian one month after referral and had a normal spermiogram. Natural and experimental EVA infection can cause ampullitis in stallions;[1,2] however, it is not known if attenuated infection following immunization with a modified-live EVA vaccine could have caused a temporary ampullitis with aspermia as seen in this stallion. It is important to mention that thousands of stallions have been vaccinated with this product and no similar side effects have been reported to the manufacturer.

References

1. Holyoak GR Giles RC, McCollum WH, et al: Pathological changes associated with equine arteritis virus infection of the reproductive tract in prepubertal and peripubertal colts. J Comp Pathol 1993;109:281-293.
2. Blanchard TL Varner DD, Hurtgen JP, et al: Bilateral seminal vesiculitis and ampulitis in a stallion. J Am Vet Med Assoc 1988;192:525-526.

Effect of medium and medium glucose concentration on equine embryo development after intracytoplasmic sperm injection

Y.H. Choi,[a] I.C. Velez,[a] B. Macías-García,[a] F.L. Riera,[b] K. Hinrichs[a]
[a]College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX;
[b]Centro de Reproducción Equine Doña Pilar, Lincoln, Argentina

In vitro equine embryo production via intracytoplasmic sperm injection (ICSI) is an important technique for both research and clinical purposes. Initially, rates of in vitro blastocyst development were low, but this improved upon culture of embryos in DMEM/F-12, a complex cell-culture medium containing a relatively high (17 mM) glucose concentration.[1,2] This study was conducted to evaluate the effect of glucose concentration during the early (Days 0 to 5) and late (>Day 5) periods of culture on blastocyst development after ICSI. We used a commercially-available one-step human embryo culture medium (GB, LifeGlobal), which contains <1 mM glucose, as a base medium. In vitro-matured oocytes were injected with spermatozoa via piezo drill, then examined for cleavage on Day 5 and for blastocyst development on Days 7 to 11. All culture media contained 10% fetal bovine serum. In experiment 1, abattoir-derived oocytes were cultured after ICSI in either DMEM/F-12, or in GB under one of two protocols of glucose addition: 0 mM for the first 5 days, then 20 mM (0-20); or 20 mM for the entire culture period (20-20). All ICSI was done with semen from one stallion (stallion EN). There was no difference among treatments in the rates of cleavage or blastocyst formation (85 to 94% and 18 to 19%, respectively, $P > 0.1$). In experiment 2, four different protocols of glucose addition (0-10, 0-20, 5-10, and 5-20) in GB were examined, using oocytes obtained by ultrasound-guided transvaginal follicle aspiration from live mares. Eight replicates of all four treatments were performed using semen from one stallion (SP). One treatment (0-20) was also performed concurrently with semen from a second stallion (HY). No differences were found in the rates of cleavage and blastocyst formation among the four glucose treatments using semen from SP (88-95% and 31-46%, respectively). However, within the 0-20 treatment, oocytes injected with sperm from stallion HY had a significantly lower cleavage rate than that for SP (63 vs. 95%; $P < 0.01$) and tended to have lower blastocyst production (23 vs. 41%, respectively; $P = 0.07$). These results indicate that glucose-supplemented GB medium can be used for equine embryo culture, and that within the parameters used in our study, glucose concentration did not significantly affect blastocyst rate. Individual differences among stallions may significantly affect embryo development in vitro.

Keywords: Equine, oocytes, intracytoplasmic sperm injection, embryo culture, glucose

Acknowledgements

This work was supported by the Link Equine Research Endowment Fund, Texas A&M University, and by Ms. Kit Knotts.

References

1. Choi YH, Roasa LM, Love CC, et al: Blastocyst formation rates in vivo and in vitro of in vitro-matured equine oocytes fertilized by intracytoplasmic sperm injection. Biol Reprod 2004;70:1231-1238.
2. Hinrichs K, Choi YH, Love LB, et al: Chromatin configuration within the germinal vesicle of horse oocytes: changes post mortem and relationship to meiotic and developmental competence. Biol Reprod 2005;72:1142-1150.

Effects of dietary zearalenone exposure on the reproductive performance of mares

Heath King,[a] Susan Bowers,[b] Nancy Shappell,[c] David Christiansen,[a] Kevin Walters,[a] Renee Jaklitsch,[a] Jennifer Tucker,[a] Richard Hopper,[a] Peter Ryan[a,b]

[a]Department of Pathobiology and Population Medicine, [b]Department of Animal and Dairy Sciences, Mississippi State University, Mississippi State, MS; [c]USDA-ARS, Fargo, ND

Zearalenone is a phytoestrogenic mycotoxin produced by the fungus family *Fusarium* that is often a contaminant of common animal feedstuffs. Its estrogenic effects in swine and reproductive disorders in cattle have been well documented, but the effect of the mycotoxin on equine fertility is relatively unknown. The objective of this study was to assess the effects of zearalenone on reproductive efficiency in healthy mares at two different concentrations. To this end, 21 mature, healthy, and reproductively sound mares (2 -16 yr) were age-matched and assigned to one of three treatment groups (n=7), control and either 2 mg (low dose) or 8 mg (high dose) zearalenone/day. Mares were fed (08:00 h/day, 0.5 kg horse pellets) using nose-bags that included a horse-treat previously soaked with zearalenone in ethanol. Treatments commenced on day of ovulation (d 0) and continued for three consecutive estrous cycles. Reproductive activity was monitored every other day by ultrasound (ovary, reproductive tract) and stallion-teasing. Serum was collected on d 0, 2, 4, 8, and 16 of each cycle for progesterone and estrogen analyses. Upon detection of a 32 mm follicle and uterine edema, serum was collected and reproductive activity examined daily until ovulation was confirmed. All mares were bred on the third estrus with at least 250 million progressively motile, morphologically normal sperm. One mare in the control group did not ovulate, while another in the low dose had an interovulatory interval of 52 days. The mean interovulatory intervals were 21.1±0.7, 25.8±4.2, and 20.3±1.1 days for the high dose, low dose, and control mares, respectively. Pregnancy rates were 7/7, 4/7, and 5/7for the high dose, low dose, and control mares, respectively. Results from endocrine analysis may provide clearer insight into the potential disruptive effects of dietary zearalenone exposure in mares. However, initial observations suggest that at these environmentally relevant doses of zearalenone, mares do not exhibit adverse reproductive effects.

Keywords: Zearalenone, mare, fertility

Persistent luteal function and spontaneous lactation in a non-pregnant mare

Justin T. Hayna
Mobile Advanced Reproductive Equine Services, LLC, Downers Grove, IL

An 18-year-old Thoroughbred mare presented in August 2009 for spontaneous lactation of more than 30 d duration. Reproductive evaluation was performed using transrectal palpation and ultrasonography. The cervix was soft, the uterus flaccid without edema, and the ovaries were without palpable abnormality. A hyperechoic structure (presumed corpus luteum) was imaged on the right ovary. Two uterine cysts, 17 mm and 11 mm, were noted near the cervix. There was no evidence of pregnancy. The udder was enlarged, fluid filled, though not inflamed; the expressed fluid was white and watery, consistent with milk. Closprotenol was administered (375 μg, IM) and on re-evaluation in 7 d, the cervix was relaxed, the uterus possessed grade III edema, and the ovaries were without palpable abnormality. The left ovary was unremarkable, and a 40 mm follicle was present on the right ovary. The uterine cysts were unchanged. Expression of the udder yielded no milk. Lactation recurred in June 2010. Estrous behavior had been noted once during 2010. The cervix was tight, the uterus was moderately toned, the right ovary was unremarkable, and the left ovary contained a 26 mm follicle and a hyperechoic structure (corpus luteum). Uterine cysts were still present. The udder was enlarged and the expressed fluid was watery and white. In an effort to find a cause for the lactation, blood samples were obtained to evaluate plasma hormone concentrations.

Reproductive evaluation and blood samples, collected via jugular venipuncture, were performed every 3 to 4 d for 49 d. Reproductive evaluation consistently demonstrated progesterone influence of the tubular tract: a closed tight cervix and a toned uterus. Throughout the sampling period, a hyperechoic structure was present on the left ovary and all follicles were less than 28 mm. Venous blood was collected into heparinized evacuated tubes, refrigerated until centrifuged, and plasma stored at -20°C until analyzed. The patient was lost to follow-up after the 49 d sampling period. All hormones were measured using radioimmunoassay. The mean ± SD for luteinizing hormone (LH), follicle stimulating hormone (FSH), progesterone and prolactin were 0.71 ± 0.23 ng/mL, 6.72 + 2.22 ng/mL, 12.81 ± 2.06 ng/mL, and 2.84 ± 1.78 ng/mL, respectively. The concentration of progesterone remained above 10 ng/mL for the entire sampling period.

In a normal cycling mare, progesterone concentrations should remain elevated for approximately16 days, followed by an interval of baseline (<1 ng/mL) concentrations. This mare maintained her corpus luteum for 49 d, as evidenced by transrectal ultrasound and confirmed by progesterone analysis. Progesterone never declined to baseline levels. The gonadotropin concentrations (FSH and LH) were consistent with diestrus. The cause of prolonged luteal function in this mare is unknown. Previous administration of cloprostenol had resulted in luteolysis. It is speculated that endogenous prostaglandin production was insufficient to induce luteolysis, and that lactation was related to prolonged progesterone exposure combined with the presence of prolactin.

Keywords: Progesterone, equine, lactation, prolactin, persistent corpus luteum

Impact of activation and subsequent antimicrobial treatment of dormant endometrial streptococci in the Thoroughbred problem mare – a descriptive field study

M.R. Petersen,[a] K. Lu,[b] M. Christoffersen,[c] J. Møller Nielsen,[d] M.H. Troedsson,[e] A.M. Bojesen[f]
[a]The Fertility Clinic, University Hospital of Copenhagen, Denmark; [b]Hagyard, Davidson and McGee, Lexington, KY; [c]Veterinary Reproduction and Obstetrics, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark; [d]Ansager Large Animal Hospital, Ansager, Denmark; [e]M.H. Gluck Equine Research Center, Lexington, KY; [f]Department of Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

The expected fertility of problem mares (non-pregnant for ≥ 3 cycles despite intensive breeding management) is reduced compared to the general broodmare population (15 to 50% vs. 80 to 85% foaling rate).[1] *Streptococcus equi* subspecies *zooepidemicus (S. zoo)* can enter an inactive/dormant state with a multifocal distribution deep within the endometrium of a chronically infected mare. As most antimicrobial compounds assert their activity against metabolically active bacterial cells, we hypothesized that activation of bacterial growth and subsequent antimicrobial treatment of dormant streptococci would increase treatment efficacy and indirectly increase the fertility of the problem mare.

A total of 64 problem mares from intensively managed stud farms in Central Kentucky during the 2011 and 2012 breeding seasons satisfied the inclusion criteria (barren ≥ 3 cycles, gynecologically normal). A low volume lavage sample and endometrial biopsy were obtained in early estrus, and a bacterial activation solution (10 mL; Bactivate) was infused into the uterine lumen. A specimen for endometrial culture (low volume lavage or guarded swab) was obtained 24 h after activation. Activation was classified as successful if culture-negative or *S. zoo*-negative (e.g. *E. coli*) on day 0 changed to culture-positive for > 5 CFUs *S. zoo* 24 h after activation. On the day following activation, mares with positive uterine cultures were treated with systemic and intrauterine antimicrobials, ecbolics, uterine lavage with or without mucolytics, and bred in the following cycle (maximum two cycles). Pregnancy was established in 53 (83%) mares. Of the 21 pregnancies established in 2011, 18 (86%) gave birth to a live foal. Foaling data from the 2012 season are pending. Since all mares were infused with the activation solution, the pregnancy rate of non-activated mares cannot be determined. The results clearly indicate that activation and subsequent antimicrobial treatment of dormant *S. zoo* in problem mares can restore the expected pregnancy and live foal rates to levels reported for the general mare population.

Keywords: Chronic endometritis, mare, *Streptococcus zooepidemicus* dormancy, activation, fertility

Reference

1. Bosh KA, Powell D, Shelton B, et al: Reproductive performance measures among Thoroughbred mares in central Kentucky during the 2004 mating season. Equine Vet J 2009;41:883-888.

Uterine horn torsion associated with a mummified fetus in a ewe

E.L. Larsonberg, L.K. Pearson, A.J. Campbell, A. Tibary
Comparative Theriogenology, Department of Veterinary Clinical Sciences, Washington State University College of Veterinary Medicine, Pullman, WA

Diagnosis of uterine torsion in small ruminants is rare, but has been described in either direction, of variable severity, and with variable numbers of fetuses.[1] This case is the first known report of uterine horn torsion associated with a fetal mummy in ewes.

A 5–year-old multiparous Icelandic ewe presented for dystocia of 2.5 hours duration. Reproductive examination demonstrated a partially dilated cervix; failure of cervical dilation and uterine torsion were considered differential diagnoses. Ultrasonography was not performed due to the duration of dystocia. A cesarean section was performed via left flank approach. A 180-degree uterine horn torsion was identified intraoperatively which contained a mid-term fetal mummy. The contralateral uterine horn contained two viable lambs which were delivered without complication. The mummified fetus, placenta, and ewe serum were submitted for diagnostic testing which was negative for infectious causes of abortion. The fetal size and development and lack of infectious agents suggest that the uterine horn torsion occurred in mid-gestation, resulting in fetal blood supply occlusion and mummification. Both live-born lambs developed high temperatures and respiratory distress several hours after birth, which resolved by 12 hours of age, and likely was due to hypoxic stress during dystocia. The ewe recovered uneventfully and was maintained on the farm for fiber production.

This case is an example of a disease that is not well-characterized in small ruminants and proposes a mechanism of non-infectious fetal mummification in the ovine. The finding of uterine horn torsion versus the more typical uterine body torsion was notable. Transabdominal ultrasonography would have been helpful in this case to identify altered uterine blood flow associated with the horn torsion and mummy as well as fetal viability. Practitioners should consider uterine torsion as a contributing factor to dystocia, especially in cases of failure of cervical dilation.

Keywords: Ovine, dystocia, cesarean section

Reference

1. Ijaz A, Talafha AQ: Torsion in the uterus of the Awassi ewe. Aust Vet J 1999;77:652-653.

Marble-induced pyometra in an Appaloosa mare

Jessica Klabnik-Bradford, Maria Soledad Ferrer, Christopher Blevins, Laurie Beard
Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, Manhattan, KS

Marble-induced pyometras have not been reported previously. This case reports a new possible complication of using intrauterine marbles to suppress estrus in mares. A 16-year old Appaloosa mare presented with a foul-smelling, suppurative vulvar discharge. No reproductive history was available. Pyometra, endometritis or vaginitis were suspected. Transrectal palpation, transrectal ultrasound and fluid cytology were performed. Transrectal ultrasound revealed a corpus luteum and a large fluid-filled uterus. A 34-mm round hyperechoic structure was noted in the left uterine horn. The cervix was firm. Cytology of the draining fluid revealed large numbers of degenerate neutrophils. Pyometra was confirmed and the presence of a mummified fetus or intrauterine marble was suspected. Uterine endoscopy was indicated to directly assess the uterine lumen, which confirmed the structure to be a glass marble. It was recommended to remove the marble during estrus and treat the pyometra. Diestrus was shortened with dinoprost (5 mg IM) and the marble was removed during estrus. Misoprostol (200 μg) was administered intra-cervically to induce cervical dilation and facilitate marble removal. The marble was manipulated towards the cervix transrectally and then through the cervix per vagina. The owners declined treatment of the pyometra. After questioning the owners, it was determined that the marble had been in place for at least two years. This case suggests that pyometra may be a complication of using intrauterine marbles for estrus suppression in mares and stresses the importance of removing the marble once estrus suppression is no longer desired. Pyometra is typically associated with cervical incompetence.[1] This mare's cervix dilated properly during estrus, and estrus was confirmed prior to presentation. It is possible that the marble acted as a foreign body or a nidus for infection. Although this mare's fertility was not tested, it would be reasonable that the pyometra could impact long-term fertility.[1]

Keywords: Pyometra, glass marble, mares

Reference

1. Pycock JF: Therapy for mares with uterine fluid. In: Samper JC, Pycock JF, McKinnon AO, editors. Current therapy in equine reproduction. St. Louis: Saunders Elsevier; 2007. p. 93-104.

Prepubic tendon rupture in late term mares - a genetic link?

Stacey Nahachewsky, Claire Card, Steve Manning, Dawne Nairn, Ilse Dedden
Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada

All breeds of horses are affected by abdominal wall tears or ruptures in pregnancy. These conditions have serious consequences and may progress to complete rupture of the prepubic tendon which can result in the loss of the mare, foal or both. Trauma, advanced age, or excessive weight due to twins may be risk factors but in most cases, no cause has been established.

In the summer of 2012 the Western College of Veterinary Medicine admitted an Egyptian Arabian broodmare that presented with bilateral abdominal herniation one day postpartum. She was stabilized and later released with a healthy foal at side. Anecdotal information from the owners revealed a similar condition in a close relative of this broodmare. Reviewing records of previous late term or postpartum abdominal herniations or prepubic tendon ruptures, it was found that since 2002, five of seven mares with prepubic tendon rupture or abdominal wall herniation have been of Egyptian Arabian descent. Researching the pedigrees of these mares revealed a common ancestry. These cases suggest there may be a genetic predisposition to prepubic tendon rupture or abdominal wall herniation in certain familial lines of Arabians. This further prompted investigation into abdominal wall thickness of normal late gestation and non-pregnant mares. The abdominal walls of 12 healthy non-pregnant mares and 13 healthy mares greater than 10 months pregnant were measured. No significant difference was found between breed groups or between pregnant and non-pregnant mares.

The hypothesis of a heritable component for the development of prepubic tendon rupture or abdominal wall herniation is still under current investigation. Focus now lies on measurements of abdominal wall thickness in Arabian mares, both pregnant and non-pregnant.

Selected references

Adams SB: Rupture of the prepubic tendon in a horse. Equine Pract 1979;1:17-19.
Dolente BA, Sullivan EK, Boston R, et al: Mares admitted to a referral hospital for postpartum emergencies: 163 cases (1992-2002). J Vet Emerg Crit Care 2005;15:193-200.
Hanson RR, Todhunter RJ: Herniation of the abdominal wall in pregnant mares. J Am Vet Med Assoc 1986;189:790-793.
Jackson PGG. Rupture of the prepubic tendon in a shire mare. Vet Rec 1982;111:38.
Kawcak CE, Stashak TS: Predisposing factors, diagnosis, and management of large abdominal wall defects in horses and cattle. J Am Vet Med Assoc 1995;206:607-611.
Ross J, Palmer J, Wilkins P. Body wall tears during late pregnancy in mares: 13 cases (1995-2006). J Am Vet Med Assoc 2008;232:257-261.

Diagnosis and treatment of a pregnant bitch with immune-mediated polyarthritis

Carla Barstow, Kelly Hall
College of Veterinary Medicine, University of Minnesota, St. Paul, MN

Immune mediated polyarthritis is a relatively common disease in dogs resulting in painful joint effusion. It is a type III hypersensitivity reaction where immune complexes deposit into joints, causing inflammation and cytokine release, thereby attracting neutrophils which cause further damage.[1] The stifle and carpal joints are most commonly affected in middle age, medium to large breed dogs.[2] Currently, glucocorticoids are the cornerstone of treating animals with immune mediated polyarthritis.[2]

A two- year-old intact female Golden Retriever dog presented to the University of Minnesota Veterinary Medical Center Emergency Services for acute onset of lethargy with shifting leg lameness and a stilted gait. Focused assessment with sonography for trauma (FAST) indicated at least three puppies with normal heart rates, approximately 34 days old. Bilateral carpal and stifle effusion was noted. An inflammatory leukogram with a mild anemia and mild hypoproteinemia, consistent with systemic inflammatory response, was present. SNAP 4Dx Plus (Canine Heartworm Antigen-Anaplasma Phagocytophilum-Platys-Borrelia Burgdorferi-Ehrlichia Canis-Ewingii Antibody Test Kit, IDEXX Laboratories, Westbrook, ME) was negative. Cytology of fluid obtained via arthrocentesis demonstrated marked non-degenerate neutrophilic inflammation with no organisms, confirming a diagnosis of immune mediated polyarthritis. Treatment options discussed with the owner included: prednisone, niacinamide and doxycycline, or delaying therapy until after whelping. Prednisone can lower gonadotropin concentration in bitches and can lead to spontaneous abortion.[3] Doxycycline, while synergistic with the niacinamide, can affect bone formation in developing puppies.[4] The owner elected niacinamide therapy only. As of this writing the pregnancy was progressing normally and the bitch's clinical signs had resolved.

Pregnant animals can be difficult to treat with the recommended drug protocols, as drugs may have deleterious effects to the unborn fetuses. This case emphasizes the importance of having multiple treatment options available and communication with owner regarding risk and benefit of various therapies for the safety of both the bitch and her unborn puppies.

Keywords: Canine, immune-mediated polyarthritis, niacinamide, pregnancy

References

1. Johnson KC, Mackin A: Canine immune-mediated polyarthritis part I: Pathophysiology. J Am Anim Hosp Assoc 2012; 48:12-17.
2. Johnson KC, Mackin A: Canine immune-mediated polyarthritis part II: Diagnosis and treatment. J Am Anim Hosp Assoc 2012;48:71-82.
3. Olson P, Johnston S, Root M, et al: Terminating pregnancy in dogs and cats. Anim Reprod Sci 1992;28:399-406.
4. Plumb DC: Veterinary drug handbook, 7th ed. Ames(IA): Blackwell Publishing; 2011.

A case of transitional cell carcinoma in the vagina

DeAnna Cotton, Robyn R. Wilborn, Aime K. Johnson, Eileen K. Jenkins, Ralph Henderson
College of Veterinary Medicine, Auburn University, Auburn, AL

A seven-year-old spayed female Wirehaired Pointing Griffon presented to Auburn's neurology service for inappropriate urination and defecation for the past year. The owner had also noted vaginal discharge. Neurologic examination and magnetic resonance imaging (MRI) were performed and no abnormalities were noted. The patient was evaluated for evidence of an ovarian remnant due to intermittent vaginal discharge and inconclusive results of an abdominal ultrasound examination. The theriogenology service attempted a vaginal examination and cytology, but the patient was extremely fractious and both were unsuccessful. These procedures were repeated successfully when the patient was under general anesthesia for the MRI. Upon digital palpation a mass was located at the junction of vestibule and vagina and pain was evident even under general anesthesia (tachypnea, tachycardia). Vaginal cytology and luteinizing hormone and progesterone assays were used in combination and revealed no evidence of an ovarian remnant. Cytology of the vaginal smear suggested a sarcoma. Vaginoscopy was performed in order to obtain a more diagnostic sample of the mass and histopathology revealed a possible adenocarcinoma.

Six days later, the patient returned for complete excision of the mass. During surgery the goal of complete excision changed to maintaining proper anatomical function of the urethra. Final diagnosis of the mass revealed a transitional cell carcinoma (TCC), papillary and infiltrating vaginal mass at the urethral papilla. The margins were incomplete, and the oncology service recommended peroxicam and mitoxantrone with fractional radiotherapy.

Urinary bladder tumors are usually TCC while vaginal tumors are more commonly leiomyomas.[1-3] The mean survival time for animals using this chemotherapy protocol was 326 days. But, the overall survival time was not superior unless mitoxantrone, prioxicam and radiation were used together.[4]

This patient was unusual because of the location of the TCC. Animals with persistent urinary problems, whether urinary tract infections or incontinence, that seem refractory to conventional therapy warrant further evaluation and a digital vaginal examination should be considered. Unexplained lower back pain may need to be explored further with a rectal and vaginal examination.

Selected references

1. Marconato L, Zini E, Lindner D, et al: Toxic effects and antitumor response of gemcitabine in combination with peroxicam treatment in dogs with transitional cell carcinoma of the urinary bladder. J Am Vet Med Assoc 2011;238:8:1004-1010.
2. Baker R, Lumsden J: The urinary tract. In: Baker R, Lumsden J, editors. Color atlas of cytology of the dog and cat. St. Louis: Mosby; 2000.
3. Sontas B, Altum E, Guvenc K, et al: Vaginal neurofibroma in a hysterectomized poodle dog. Reprod Domest Anim 2010;45:1130-1133.
4. Poirier V, Forrest L, Adams W, et al: Piroxicam, mitoxantrone, and coarse fraction radiotherapy for the treatment of transitional cell carcinoma of the bladder in 10 dogs: a pilot study. J Am Anim Hosp Assoc 2004;40:131-136.

Recurrent seminal vesculitis in a stallion

A.K. Daniel, A.K. Johnson, R.R. Wilborn
Department of Clinical Sciences, College of Veterinary Medicine, Auburn University, Auburn, AL

This case demonstrates the challenges of identifying and treating a reproductive infection in a stallion. A seven year old Gypsy Vanner stallion presented for oligiospermia. Initial collection revealed severe leukospermia. The epididymis of the right testicle was enlarged and firm. On transrectal ultrasound, the right seminal vesicle was thickened and filled with flocculent fluid. Bacterial culture of the ejaculate revealed *Pseudomonas aeruginosa*. Systemic antibiotic therapy was initiated with enrofloxacin (7.5 mg/kg IV q24 hrs for seven days).

Due to an abnormal right epididymis and lack of improvement in semen quality, a hemicastration was performed after four days of treatment. Histopathology revealed a fibrosed epididymis and degenerate testicle with absence of mature spermatids. The stallion was discharged for 60 days of sexual rest.

On subsequent examination, leukospermia was still present. A urethroscopy showed the right seminal vesicular gland was inflamed and filled with thick, mucopurulent material. A sample was collected for culture and again *Pseudomonas aeruginosa* was isolated. Both seminal vesicles were lavaged with saline and infused with 1.5 g of ticarcillin daily for five days. Systemic therapy with enrofloxacin was instituted. Following treatment and one week of sexual rest, the leukospermia returned, and a culture of the ejaculate was again positive for *Pseudomonas aeruginosa* with the same susceptibility pattern. Seminal vesicular lavages and ticarcillin infusion were again performed daily for seven days, and systemic enrofloxacin was initiated for a period of thirteen days. Following this treatment, a culture of the ejaculate was negative for pseudomonas. The stallion remained negative one month after the last treatment and was discharged.

This case illustrates the difficulty of treating *Pseudomonas aeruginosa* in the stallion reproductive tract. If this stallion remains free of infection, his prognosis for fertility is excellent. Due to recurrent seminal vesiculitis despite treatment, this stallion is at risk for re-infection which may require more aggressive therapy.