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Veterinarians Dedicated to Animal Reproduction

Clinical Theriogenology

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The American College of Theriogenologists

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Clinical Theriogenology

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The Society for Theriogenology
and
The American College of Theriogenologists

Mission Statement

The purpose of *Clinical Theriogenology* is to publish in a timely manner peer-reviewed information relevant to the clinical practice of theriogenology for veterinary practitioners, academic clinicians, and veterinary students. The journal will be the means by which the Society for Theriogenology (SFT) publishes the proceedings of its Annual Conference and Symposia.

Scope of the Journal

Clinical Theriogenology will be broad in scope and manuscripts published will be in the following categories:

- Research reports
- Reviews of current literature
- Clinical reports
- Innovative techniques
- Book reviews
- Letters to the editor
- Editorial opinion
- News from the Society for Theriogenology and the American College of Theriogenologists

Publication Schedule

The regular issues will be published quarterly. On occasion, the Editorial Board will consider issuing a Festschrift to honor eminent theriogenologists.

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Manuscripts are accepted for consideration with the understanding that they have not been published elsewhere (except in the form of a brief abstract) and are not simultaneously under review by another journal. The manuscript must be in English (American spellings), and follow the Uniform Requirements for Manuscripts Submitted to Biomedical Journals (http://www.icmje.org). The following guidelines are applicable:

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- Font: Times New Roman; size 12
- Left-justified
- 1" margins at the top, bottom, and sides of each page
- All pages (including the title page) are to be numbered consecutively
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The general format for scientific manuscripts is as follows:

- Title page: Contains the title of the paper and the first and last names of each of the authors; middle initials are optional. Specify the corresponding author and her/his contact information (mailing address, telephone and fax numbers, e-mail address). Do not list academic degrees or specialty board certification. State the sources of funding (if any) and any meetings at which the data were presented.
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  Martinez MF, Adams GP, Kastelic JP, et al: Induction of follicular wave emergence for estrus synchronization and artificial insemination in heifers. Theriogenology 2000;54:757-769.

  Book (personal author)

  Johnson SD, Kustritz MVR, Olson PNS: Canine and feline theriogenology. Philadelphia: Saunders; 2001. p. 7.

  Book (edited, multi-author)

  Woods GL, Hallowell AL: Management of twin embryos and twin fetuses in the mare. In: McKinnon AO, Voss JL, editors. Equine reproduction. Philadelphia: Lea and Febiger; 1993. p. 532.

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Title of Case
Authors of case. Please indicate corresponding author by * (after the author's name)
Summary. Up to 150 words summarizing the case presentation and outcome
Background. Why is this case important?
Case Presentation. Presenting features, pertinent medical history, herd history (if applicable)
Differential Diagnosis. (if relevant)
Treatment.
Outcome .
Discussion. Include a brief review of similar published cases; how many other similar cases have been reported?
Learning points. Three to five bullet points
References. Vancouver style
Figure/photo captions. (if any)

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Student Case Presentations

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*.

The 2012 Bartlett Address
It's all about the people

Ted F. Lock
Professor Emeritus, University of Illinois, Bloomington, IN

I would like to thank the selection committee for the great honor being selected the 2012 recipient of the Bartlett Award. I would also like to thank Dr. Augustine Peter for the nomination. When Dr. Hopper called me in February, my first reaction was "there must be a mistake". Never in my wildest dreams did I think I would ever be the recipient of this prestigious honor. It also made me realize I was getting closer to the "golden years". When it finally sank in that he was serious, it made me start thinking of all the people who had influenced my life and the path to this podium, and in my professional life, many of those people have stood at this podium. I want to take this opportunity to express my appreciation to all of the people who have influenced my life.

Family first

I must start with my family. I grew up on a small livestock farm in Missouri. My father was a true stockman. He taught me the importance of "good feed and clean water". He also operated a livestock auction, so I had many opportunities to learn how to work around large animals. I learned how to castrate, vaccinate, dehorn, etc. while working with the veterinarians at the auction. Pregnancy examinations and testing cattle for brucellosis convinced me that I wanted to be a veterinarian. My mother made sure I did my homework and stressed the importance of a good education.

I am most grateful to my wife, Diann, who has been my rock throughout most of my life. Her love, support and tolerance have been instrumental in any success I may have experienced. We married after my second year in veterinary school and tomorrow we will celebrate our 43rd wedding anniversary. I would also like to thank my daughter Mary, who along with her mother, really influenced my understanding of the human-animal bond. "It turns out that dogs can live in the house". Attending horse shows with my mother- and father-in-law gave me my first appreciation of quality horses.

University of Missouri

I entered the University of Missouri-Columbia College of Agriculture in the fall of 1965. I got a job as a laboratory assistant in the Agricultural Biochemistry department and got my introduction to research in ruminant nutrition. Working with rumen fistulated cows was more interesting than cleaning lab ware and the path toward veterinary school continued.

I was fortunate to be accepted to the School of Veterinary Medicine in fall of 1967. The professors in basic sciences were excellent and gave us a solid foundation to enter clinics. The clinicians who influenced me most were Dr. C. J Bierschwal, Dr. Charles Martin, Dr. Joe McGinity, Dr. Walter Threlfall and Dr. Ed Mather. Bull breeding soundness examinations were a significant part of the caseload and we were encouraged to become members of the "American Veterinary Society for the Study of Breeding Soundness" aka the "Society for Theriogenology". I joined the Society when I graduated in 1971 and have maintained my membership ever since. It has been one of the most important Associations in my professional life.

Dr. Bierschwal had done a sabbatical at the University of Utrecht and introduced us to the Utrecht fetotome, a very useful instrument for the type of obstetrical cases we often dealt with (a beef cow with a dead, emphysematous fetus). One of my classmates, Dr. James Snodgrass had experience as a machinist and working with Dr. Bierschwal, manufactured the Utrecht fetotome for marketing in the United States.

We did not have year round clinics in those days and I was hired as a student intern in the Food Animal Section between my third and fourth year. That gave me an opportunity to work closely with the clinicians and was an extremely valuable experience.

A Saturday morning in May, 1971 changed the course of my career. I was paged to come to the Department Head office. Dr. Charles Martin was the acting head of the Department. He said "Lock, we've been watching you and we think you have potential as a teacher", and offered me to opportunity to

stay on as an instructor. That was a complete shock to me. I was a fairly average student and an academic career was the farthest thing from my mind. My plan was to go into large animal practice and I had just committed to a practice position. But that statement from Dr. Martin planted the seed of what would become the best career course I could ever imagine. I joined a very busy primarily large animal practice and learned a lot during that first year. We had a nice mix of beef and dairy cattle and horses. The most famous (infamous) animal I worked with during that year was a young Quarter Horse stallion named "Impressive". Little did I know the impact he would have on the horse industry.

University of Illinois

The idea of a career in academia lingered in my mind during that year and in 1972, I accepted a position as an instructor in the ambulatory section at the University of Illinois. Illinois did not have a residency program in theriogenology but that position allowed me to enter a graduate program that would eventually qualify me to take the ACT examination.

The person who most influenced me in the early years at Illinois was Dr. Bruce Brodie. His philosophy was that a good caseload was important to a good clinical teaching program and being a clinician at heart, I was on board with that philosophy. My assignment at Illinois was to develop the equine field program and I was fortunate soon after I arrived to meet Leo Daly, who was starting a Standardbred breeding farm. He was receptive to the idea of allowing students to palpate mares. Artificial insemination was beginning to be accepted by more breeds and this association allowed me to gain a lot of experience and to teach students the "art" of rectal palpation and other procedures involved in a breeding farm operation.

Dr. Jim Eagleman and Dr. Don Lingard (large animal clinic faculty) were teaching the theriogenology course until 1975. During that year, Dr. Eagleman retired and Dr. Lingard took a position at Lousiana State University and I was asked to take responsibility for coordinating the theriogenology course. I was still working on my graduate program but this was an assignment I could not turn down. I completed my master's degree in 1976 and became an assistant professor in Veterinary Clinical Medicine.

I submitted my credentials to the American College of Theriogenologists and they were accepted and in 1977, I took the ACT Board examination. Dr. Bill Wagner proctored my examination. At that time, the examination was all essay questions and after writing for twelve hours, I was sure that I would be repeating in 1978 but was fortunate to be invited to St. Paul, MN for the oral examination. What an honor it was to become a Diplomate of the American College of Theriogenologists!

Dr. Randy Ott had joined the faculty so Drs. Brodie, Ott, and I team taught the theriogenology course. In 1978, Dr. Borje Gustafsson became the department head and we officially formed a theriogenology section. His support for research gave the section a much needed boost in developing the residency and graduate program. Drs. Wagner and Gustafsson were instrumental in attracting the "International Congress on Animal Reproduction" to the University of Illinois in 1984.

In 1980, I was asked to serve on the ACT examining committee. It did not take very long to realize how much work had gone into the examination process. It was an honor and a great learning experience to serve with people like Drs. Stanley Dennis, Steve Roberts, Larry Evans, Don Schlafer, Larry Rice, Howard Whitmore and many more very dedicated people. Many hours were spent reading the essays of candidates and developing questions for the oral examination.

In 1986, I became secretary, so my term on the examining committee was extended another three years. The College did not have an executive secretary, so the secretary was responsible for maintaining all the correspondence with candidates and all the paperwork associated with the examination. Dr. Howard Whitmore served as secretary before me so transferred the boxes of files down the hall to my office.

A concern during that time was assuring that the examination was fair and objective, so a lot of time was spent transitioning from long essay questions to shorter and more defensible questions. The evolution of the examination process and the hard work of the committee made it possible for candidates to find out their results in hours instead of weeks.

I owe a great deal of gratitude to Don Ellerbee and Jan Weiler for their help in the logistics of the examination process. They worked out the details of room availability and many other details that made the process go smoothly.

In 1989-90, with the help of Dr. David Galloway, I was fortunate to arrange a sabbatical at the University of Melbourne, working with the McKinnon Project, a production medicine group focusing on sheep and beef cattle. It was a rejuvenating experience and gave me a broader perspective on the role of theriogenology in production medicine. I returned to Illinois re-energized and with a new appreciation for what I felt was the greatest career path I could ask for- teaching veterinary students.

The accidental teacher

For a guy who had once envisioned himself in a career in private practice, I am grateful to have had the good fortune to stumble into a career in teaching. I have learned that one cannot always plan where life will take you, but the path it took me on has been incredible. One of the most rewarding aspects of my career at Illinois was seeing students develop their clinical skills. Watching the evolution of each class from the day they entered clinics to graduation was fascinating. The "chaos" of a new group of senior students early in the year learning to work as a team to make the clinic operate smoothly was impressive to watch. Leaders evolve in this process and many former students I had the privilege of knowing have gone on to make significant contributions to the field of Theriogenology.

During my term as ACT president, I was impressed by the continued involvement of the founding members. Drs. Bartlett, Roberts, Zemjanis and Parker wrote letters of encouragement and suggestion to enlarge the list of honorary membership in the ACT. It was an honor to have the support and friendship of people who I had so much respect for throughout my professional career.

The changes that have occurred in the field of Theriogenology during my career have been nothing short of phenomenal. When I graduated in 1971, "high tech" was the electroejaculator. Products and technologies that we use every day were unknown just 40 years ago. Prostaglandin F2-alpha, gonadotropin releasing hormone, sexed semen embryo transfer, ultrasound, in vitro fertilization, follicular aspiration are just a few of the advances that allow us as theriogenologists to provide high tech services to our clients. The Society and College have also undergone tremendous changes. Among the many changes, one of the most notable is in the publications. Education has always been a major focus of the Society and College and it has evolved from the early days when the publications were "the manuals" to the "Fact Sheets" and evolving to "Clinical Theriogenology". The evolution of the Society from primarily a large animal focus to include companion animals, zoo animals, etc. has stayed consistent with the founding members' desire to maintain theriogenology as a comparative science.

I would like to recognize Franz Management Services and Dr. Charles Franz for the service they have provided the Society and College. Their efforts have been important in giving our specialty greater visibility.

In 2005, I returned to private practice and I must admit that I have missed the daily interaction with theriogenology colleagues. I am looking forward to the meeting here in Baltimore, reconnecting with old colleagues and meeting new people who are contributing much new information to our exciting field. Theriogenology has been a major part of my life, and I cherish the friendships that I have developed over the last 40 plus years as a member of the Society and College.

Thank you for your patience in listening to the ramblings of a guy who is getting a little "long in the tooth". I wish the younger colleagues the best as they continue to advance theriogenology. I'll end with a quote from Garrison Keillor. "Be well, do good work and keep in touch."

Theriogenology liability claims: all creatures great and small

Nina Mouledous
AVMA PLIT Trust Representative, Chicago, IL

Veterinary medicine remains one of the most highly regarded occupations and yet there are challenges facing the profession. One out of 19 veterinarians will encounter an allegation of malpractice or a license complaint every year. This presentation will focus on actual claims associated with reproduction, ranging from negligence allegations, bailee claims and semen and embryo storage claims.

Malpractice is defined as the failure of a veterinarian to use such reasonable skill and diligence as are ordinarily expected of careful, skilled and trustworthy veterinarians. Veterinarians specializing in theriogenology are held to a performance level equal to that of others in their field. This performance level is called "Standard of Care", which is what other veterinarians would find reasonable for a particular situation.

There is a distinct difference between a professional liability (PL) claim, a bailee claim, semen and embryo storage claim and a board complaint (BC). A malpractice claim is an allegation of veterinary medical negligence. Veterinary Medical Board (VMB) complaints are complaints against your veterinary license and may or may not be in conjunction with a complaint of malpractice.

The most common situations that prompt claims involve miscommunication, adverse events and unfortunate outcomes (without negligence), accidental loss or injury to animals while boarding, collection procedures, economic loss, human injury, and negligence (mistakes and errors). Common situations that arise with claims associated with reproduction are small animal ovariohysterectomies, cesarean sections, equine rectal tears, pregnancy missed diagnosis, semen collection injuries, semen and embryo storage claims and more.

A bailee claim (professional extension), is associated with damage or injury to the animal while it is in the veterinarian's care, custody and control, but not involving a veterinary incident. Professional extension coverage is needed for practice owners, not associates. Negligence does not have to be proven with bailee claims.

Semen and embryo storage claims are a result of direct physical loss of the embryos or semen while in the storage tank, during transported and while in the veterinarian's care, custody or control. This exposure is not covered by the malpractice policy and is specifically excluded. Embryo and semen storage coverage picks up where malpractice coverage ends. Both coverages are important to ensure that if damage to the embryos or semen occurs during the collection and processing phase, or the storage phase, the veterinarian is protected.

Information provided in this presentation is obtained exclusively from the AVMA-PLIT sponsored professional liability program.

Keywords: Malpractice, communication, bailee

Empathy: What's all the fuss?

Kathleen Bonvicini
Institute for Healthcare Communication, New Haven, CT

Americans have increasingly scrutinized the quality of their healthcare, and for the human **and** animal loved ones in their lives. There is strong evidence in both human and veterinary medicine that how healthcare professionals communicate has a significant influence on the quality and efficiency of patient care delivery. The backbone of this communication is based on the honoring of relationships (human-animal relationships, client-veterinary practitioner relationships, veterinary team relationships) where empathy is valued and practiced by all. Providing communication training with empathy at its core is a wise investment that can lead to satisfied clients and health care team members, healthy patients, and a successful practice. For example, the predominant decision for patients pursuing litigation against their physicians is the perception of a lack of caring.[1-3] Similarly, reports from the AVMA Professional Liability Insurance Trust[4] report that approximately 60% of veterinary claims are due to miscommunication with lack of caring being a common theme. An underlying premise is that improved empathic communication among members of a veterinary team, between practitioners and clients, and even between veterinary specialists and referring veterinarians will cut down formal complaints and litigation and enhance outcomes for all. This keynote presentation will provide an overview of the role of empathy and compassion in veterinary medicine, with references to research evidence, the landscape of medical training, and empathy skill awareness and development through video case study.

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Control of mammary gland function in the bitch and queen: a review

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Introduction

Lactation can be divided into the consecutive stages of mammogenesis (development of mammary tissue), lactogenesis (production of colostrum and first milk) and galactopoiesis (maintenance of milk production under suckling and/or milking stimulus). Mammary growth and development, milk production, milk ejection and mammary involution are under the control of different hormone systems: progesterone and growth hormone act synergistically to cause mammary gland development, prolactin acts towards the end of pregnancy to start lactation and oxytocin causes milk letdown. Once suckling stimuli cease, insulin-like growth factor-I (IGF-I) is secreted in large amounts causing mammary gland involution, following which the mammary gland remains in a quiescent state for a few months. The control of different mammary functions relies on understanding how these different hormone systems and their stimulating/inhibiting factors exert their action. This paper will review the endocrine control of mammary functions in bitches and queens, as well as pharmacological treatments which can be used by small animal clinicians to treat bitches or queens with hyper- or hypo-function of the mammary gland. As the literature on physiology of the mammary gland of small animals and its normal or abnormal functions is very scant (canine) or almost non existant (feline) - with the only exceptions of mammary tumors or mastitis - the reader should be aware that a) majority of the information presented in this paper is drawn from work done (mostly) in humans or other animal species, and b) the majority of clinically relevant conclusions are extrapolated from data coming from humans or other animal species. However, it is noteworthy that mammary gland function is fairly similar across species, as it can be inferred by similarities in the action of endocrine drugs on lactation in different animal species as well as humans. Conditions such as mastitis or mammary neoplasia are beyond the scope of this paper and will not be discussed here.

Keywords: Mammary gland function, antiemetic, antipsychotic, dopamine agonists, sertonin antagonists

Mammary physiology

Mammary growth

Mammary proliferation starts at birth and is minimal (proportional to body growth) until puberty, when a rapid extension and branching of the duct system starts. Thereafter, the degree of mammary growth depends on the stage of the reproductive cycle, being maximal during gestation, early postpartum and in general during steroid-regulated phases, and minimal during anestrus. Growth is generally characterized by duct extension and branching followed by proliferation of lubules and alveoli, with the proportion of parenchyma to stroma increasing exponentiallysuch that in early postpartum period, the mammary gland is composed of a network of lobes and lobules separated by fine septa of connective tissue. Unlike what used to be thought, mammary growth is not limited to gestation but can continue also at the end of pregnancy and into the early stages of lactogenesis.[1]

The role of sex steroids in regulating mammogenesis is very similar in different species, and is characterized by the pivotal actions of 17-b-estradiol (E2) and progesterone (P4) in causing proliferation of the ductal and lobulo-alveolar components of the mammary gland, respectively, and in maintaining the mammary epithelium in a steady state (the so-called "survival action").[2,3] Ample clinical as well as experimental evidence clearly indicates the importance of estrogen signalling for normal mammary development: mammary growth (particularly ductal growth) does not occur in castrated individuals or in mice deprived (knockOut = NO) of the estrogen receptor, whereas it resumes following estrogen administration. In these estrogen receptor NO mice, complete mammary ductal growth requires administration of E2, growth hormone (GH) and adrenal corticosteroids; if P4 and prolactin (PRL) are added to this combination, normal lobulo-alveolar growth resumes.[4,5] In the postpuberal bitch, GH plays a role in the development of mammary tissue acting in concert with GH induced IGF-I and some of the IGF-binding proteins;[6] lobulo-alveolar growth and development

mainly occur during the second part of the canine luteal phase, with maximal development coinciding with the highest plasma PRL concentrations, a well-established feature of the second half of the canine diestrus.[7] Canine GH secretion, arising from foci of hyperplastic ductular epithelium of the mammary gland, is influenced by P4[8] and occurs during phases of high serum P4 concentrations (such as during the first half of the luteal phase in the bitch).[7,9]

Milk production

Once lobulo-alveolar growth has occurred, alveolar epithelial cells acquire the capacity to secrete milk through organellar and biochemical differentation. This stage is normally reached when parturition approaches, as typically the first milk secretions can be observed prior to delivery. Soon after parturition and for the first few days a thick, often yellowish milk is produced (colostrum). This phase is normally referred to as lactogenesis, while the maintenance of sustained milk production for weeks or months thanks to suckling (or milking) is called galactopoiesis. During lactogenesis and galactopoiesis, PRL and GH play a fundamental role in controlling milk production in most species studied, including dogs and cats.

While the influence of GH dominates over that of PRL in ruminants, PRL is very important for lactogenesis and galactopoiesis in rodents, humans as well as dogs[10] and presumably cats. In the dog, pituitary secretion of PRL is modulated by both stimulatory and inhibitory signals: dopamine is the main inhibitory factor, while several substances, including serotonin and thyrotropin-releasing hormone, have PRL-releasing properties.[11] Progesterone has an important modulatory effect on canine PRL secretion: when P4 concentrations are high, PRL is low or non-detectable; when P4 decreases after having been at high concentrations for a prolonged period (such as at the end of diestrus), PRL secretion is stimulated and a peak occurs. This is confirmed by the observation of high PRL concentrations in pseudopregnant as well as periparturient bitches (as both conditions require a drop on serum progesterone). Also, in pregnant bitches, PRL rises around day 35 after the LH peak;[10] such a rise occurs at a time when serum P4 starts to decrease, confirming the importance of P4 in modulating PRL release from the pituitary. No progressive increase in PRL secretion occurs in non-pregnant bitches at the same time interval after the LH peak.[10] However, PRL concentrations rise in non-pregnant dogs at the end of diestrus causing pseudopregnancy. The importance of P4 in regulating PRL secretion is further confirmed by:

a) the capacity of progestogens to eliminate clinical signs of pseudopregnancy by decreasing PRL levels; PRL will rebound once progestogen treatment is withdrawn, which would be expected as it escapes P4 regulation. For this reason progestogens should not be used for the treatment of pseudopregnancy.
b) the fact that administration of a progesterone-receptor antagonist to pregnant bitches results in highly elevated plasma prolactin levels.[12]

At the mammary level, PRL and GH induce functional differentiation through milk protein and fatty acid synthesis, with the transcription of several milk protein genes on the mammary epithelial cells being significantly increased during mid-lactation as compared to the onset of lactation.[13] The importance of PRL for stimulating lactogenesis and galactopoiesis is confirmed by the effectiveness of PRL-releasing drugs (see below). Unlike for PRL receptors, there is little evidence of the presence of GH receptors at the mammary level. Growth hormone is found in canine and feline mammary gland tissue and secretions (particularly pre-partum and in colostrum) at high concentrations, up to100-1000 times those in plasma;[6] however, canine fetuses do not show any evidence of increased GH levels, and there is no correlation between milk GH and neonatal GH due to poor absorption of GH at the gastrointestinal level.[14,15] Therefore, the action of mammary GH on mammary growth and secretion as well as the role of milk GH remain unclear in the bitch as well as in other species.

Oxytocin plays an important role in milk ejection by causing powerful contractions of the myoepithelium surrounding alveolar ducts. Its release from the hypothalamus (supraoptic and paraventricular nuclei) is modulated by sensory stimuli arising from suckling at the teats. It is currently believed that oxytocin does not interfere with the process of milk production,[16] but rather exerts its action on milk letdown. Its indications in human and veterinary include only obstetrical problems (dystocia) and the treatment of postpartum hemorrhage. The claim that it may help to

increase milk yield are so far unsubstantiated, as no connection has ever been established between oxytocin and PRL secretion.

In dogs, peak lactation is assumed to occur around the third to fourth week postpartum as puppies do not start eating semisolid food until their deciduous teeth erupt at 21-35 days of life. Normal puppy growth is generally supported by the dam's milk up to four weeks postpartum, after which reduced growth will occur if supplemental food is not provided. At peak lactation, daily milk production is approximately 1.7 kg milk/day in German sheperds and 1.0 kg/day in Beagles.[17]

Mammary involution

Once the suckling/milking stimulus is withdrawn, the mammary gland starts a gradual involution process through which it reverts back to a state of development only slightly greater than what existed at puberty. Such regression (which is normally faster if suckling/milking ceases in early lactation) is due to withdrawal of galactopoietic hormones with consequent decrease in the expression of genes responsible for milk synthesis, and therefore a decrease in milk secretion. The immediate drop in the concentrations of PRL and GH once milking or suckling stimuli cease is a mechanism demonstrated in rodents and confirmed in ruminants.[18,19] The involution effect is due to the lack of anti-apoptotic action which PRL exerts on mammary epithelial cells, and is directly proportional to the magnitude of decrease in PRL. In lactating Beagle bitches, PRL reaches its peak concentration shortly after delivery, after which it plateaus for a few weeks and then starts a gradual decrease reaching basal values around two months post-partum.[10] Canine mammary involution starts around the end of the second month of lactation.[20] In rodents and in ruminants, PRL is regarded as one of the principal endocrine signals in controlling and preventing mammary cell death. In fact, its administration following litter removal in mice delays mammary apoptosis, and its decrease following use of anti-PRL agents rapidly induces apoptosis and cell loss associated with a consistent reduction in milk yield. These observations, combined with the well known involutionary effect of antiprolactinic agents on the canine and feline mammary glands,[21,22] probably indicate that a similar mechanism occurs in small animals as well. In addition to the regulatory role of sex steroid hormones on mammary function, a wealth of novel information has been obtained on the role of E2 and P4 receptor expression and their cross-talk with the receptors for some growth factors (such as epidermal growth factor, tumor necrosis factor alpha, etc.) during lactation as well as mammary involution in experimental animals. However, these mechanisms have not been studied in carnivores and their relevance in clinical practice remains to be determined.

Pharmacological control of mammary function

Control of mammary function normally refers to stimulation or inhibition of lactogenesis and galactopoiesis, as both processes can be controlled to a great extent in humans and animals. In domestic animals, the endocrine mechanisms controlling cessation of milk production and mammary involution are more clearly defined than those controlling mammary growth and lactation. Because of a) the pivotal role played by PRL in promoting mammary growth and milk secretion, and b) the availability of PRL-lowering drugs for use in small animals, it is relatively easy to inhibit mammary function thus stopping lactation. Conversely, it is not as easy to stimulate mammary growth and lactation, particularly in bitches and queens.

Increasing milk production

Conditions in which it is necessary to increase milk production in bitches and queens include insufficient or absent milk production (generically referred to as hypogalactia or agalactia, respectively) due to inadequate mammary development during gestation. Milk production may however be lower than normal despite normal mammary development, in which case the cause of abnormal lactation is presumably due to failure of endocrine mechanisms responsible for lactogenesis and galactogenesis to activate, thereby resulting in decreased or absent PRL secretion. As with most other hormonal secretions, stress will interfere with PRL release from the pituitary; therefore, causes such as mastitis, metritis, endotoxemia, systemic illness or psychological problems may be responsible for lack of onset of lactogenesis and/or galactogenesis. Agalactia is a rather obscure condition in bitches and queen, while it has been extensively studied in women. Milk production in breastfeeding mothers can be increased using a variety of drugs called galactogogues. These include

antiemetics such as metoclopramide and domperidone; antipsychotics such as chlorpromazine and sulpiride; hormones such as oxytocin, growth hormone and medroxyprogesterone acetate. The human literature will be briefly reviewed here as the treatment of agalactia in small animals relies entirely on drugs of the human pharmacopeia.

Antiemetics. Metoclopramide is a central nervous system (CNS) dopamine D2 receptor antagonist used as a human antiemetic drug. Recommended dosages for galactogenic effect in women are 10-15 mg/day TID, per os for 3-4 weeks. Its antagonizing action on the main PRL inhibitor dopamine causes a powerful, albeit indirect stimulus to PRL release[23] with reported high efficacy rates especially when metoclopramide is associated with oxytocin nasal spray. In lactating women, metoclopramide is transferred to breast milk where it quickly becomes more concentrated than in plasma (milk-to-plasma ratio of 1.8:1), although this is not regarded as critical for babies since the milk level is below pharmacological doses. Metoclopramide acts also as an antagonist of serotoninergic receptors (although this does not prevent PRL-releasing action), and has some cholinergic effects on smooth muscle. Maternal side effects include tiredness, headache, anxiety, nervousness and intestinal disorders. At higher doses (2-5 mg/kg) the drug may penetrate the blood-brain barrier and extrapyramidal signs (anxiety, agitation, movement disorders, dystonic reactions, ataxia) are reported.

Metoclopramide is also normally used in dogs as an antiemetic drug at oral dosages between 0.2-0.4 and 1-2 mg/kg divided in 2-3 administrations.[24] It has been used also to stimulate canine PRL secretion, although scientific data with pre- and post-treatment PRL concentrations are available only for male dogs, in which a significant increase in serum PRL concentration is reported following treatment with 0.2 mg/kg 3 times daily.[25] Use of metoclopramide in bitches with agalactia is anecdotal, with protocols varying from low (0.2-0.5 mg/kg SC or PO, BID or TID[26,27]) to high dose regimens (1-5 mg/kg beginning PO or SC, every 6-8 hrs[28]). Efficacy seems to be adequate with (subjective) improvement of milk production in ≥50% of cases, although no data on PRL concentrations pre- and post- treatment are available. Extrapyramidal signs may occur in canines, and are a concern in nursing bitches, therefore higher dosages should be avoided. Improvement in milk production is generally noticeable within 24-48 hours. To minimize side effects, it is advisable to start at a lower dose (0.5 – 2 mg/kg/day divided TID) for the first 24-48 hours and then if there is no improvement gradually increase the dose every other day until an effect or abnormal clinical signs are noted, at which point the dose is dropped to the prior day's dose or discontinued. Extrapyramidal signs are much more common at doses above 2 mg/kg/day. Bitches should be monitored carefully when being treated with metoclopramide to ensure injury to the pups does not occur. Treatment should be continued for at least two to three days beyond when milk production appears to be resulting in adequate daily weight gain for the pups without supplementation.

Domperidone is a peripheral dopamine receptor antagonist developed as an antiemetic agent and used for the treatment of nausea and vomiting. In women, domperidone significantly increases PRL secretion thereby enhancing breast milk production, and is therefore used (off-label) as a galactogogue in most Western countries.[23] In 2004, a few cases of cardiac arrythmia and sudden death were observed in US patients suffering from cancer and with low potassium who were receiving high IV doses of domperidone concurrently with chemotherapy. This prompted an FDA warning that breastfeeding women should not use domperidone, after which the drug was subsequently withdrawn from the US human market. Subsequent research has shown that domperidone is safe when used by lactating mothers.[29] The maximum approved treatment protocol of domperidone in lactating women is 20 mg given four times daily, although most authors advize using doses of 10 mg orally TID for one to two weeks. However, the minimum effective dose and the minimum duration of therapy have not been identified yet. Domperidone causes a significant increase in serum PRL concentrations and milk production in treated vs control mothers, which has been estimated at 75% in a recent meta-analysis.[30] Unlike metoclopramide, domperidone is less permeable to the blood-brain barrier and is transferred in moderate quantities to maternal milk (milk-to-plasma ratio of 0.2-1.1), due to its high molecular weight and its 90% binding to plasma proteins.[23] No side effects are reported in infants of mothers taking domperidone, while side effects in mothers include oral mucosal dryness, skin eruption, itch, headache and gastrointestinal disorders; extrapyramidal effects have been observed (dystonia) but are rare. No difference in milk quality of mothers treated with domperidone is reported, except for significant increases in carbohydrate and calcium.[29]

Early experimental use of domperidone has been reported in the dog, with data on pharmacokinetics[31] and excretion and metabolism[32] in Beagle dogs. However, there is a lack of scientific as well as anecdotal information on clinical use of domperidone in small animals with low milk production. This is surprising given the positive results and the lack of side effects of this drug making it probably the best treatment for increasing milk production in lactating mothers. Domperidone is known among small animal clinicians by "word of mouth" to be effective in improving milk yield at doses of 1.5-2.0 mg/kg in queens, and 2.2 mg/kg in bitches, per os for one to three weeks. Treatment should be continued for at least two to three days beyond when milk production appears to be resulting in adequate daily weight gain for the pups without supplementation. In our experience, results of domperidone in increasing milk production in agalactic bitches and queens appear to be positive, better than those obtained with metoclopramide and devoid of extrapyramidal effects. Diarrhea is the most common side effect in the bitch, although there are anectodal reports of behavior changes in some bitches being medicated.

Antipsychotics. Chlorpromazine is an antagonist of D2 dopaminergic hypothalamic receptors, commonly used for the treatment of human psychosis including schizophrenia and depression. It is considered the prototype of the phenothiazine class of drugs. Its action on dopaminergic receptors causes PRL release, which is the reason for its off-label use in breastfeeding mothers. It is transferred to milk in low quantities (milk:plasma ratio of 0.5), and its recommended dosage for galactogogic effect is 25 mg orally TID for one week.[23] Chlorpromazine has a wide action on different CNS receptors producing also anticholinergic, antihistaminic, as well as weak antiadrenergic effects. The main side effects of chlorpromazine in psychotic patients (who are treated with higher doses than lactating mothers) are mostly due to its anticholinergic properties and include sedation, slurred speech, dry mouth, constipation, urinary retention, possible lowering of the seizure threshold, increased appetite and impaired glucose tolerance leading to increase in weight. Not much is known about side effects of chlorpromazine in breastfeeding mothers and their infants,[33] although lethargy, sleepiness and reduced activity have been reported in a few babies.[34] For these reasons, the American Academy of Pediatrics (AAP) in 2001 stated that chlorpromazine is included in the list of drugs whose effects on nursing infants may be of concern,[34] and some authors advice monitoring of infants whose mothers are under chlorpromazine treatment.[35]

In small animals, chlorpromazine is used as a second choice antiemetic drug when metoclopramide does not work and blood pressure is normal.[36] Suggested antiemetic dosage is 0.2-0.5 mg/kg every 6-8 hrs. Only anecdotal information on the use of chlorpromazine in cases of agalactia is available for small animals.[37] Some authors advize the use of acepromazine at 0.125-0.5[38] or 0.5-2.0 mg/kg,[39] SC 2-3 times/daily. No data on clinical efficacy in bitches or queens as well as milk:plasma ratio of transfer are available for this drug.

Sulpiride is a substituted benzamide used as an antipsychotic drug for the treatment of human psychosis including schizophrenia and depression. It is a strong antagonist of serotoninergic receptors as well as of muscarinic, alpha-adrenergic and histaminic receptors. Its administration (off-label) to breast-feeding women in galactogenic doses of 50 mg orally 2-3 times/day for one to four weeks produces a strong PRL-releasing effect with PRL reaching serum concentrations which may be up 90% higher than in the control group and infants of treated mother gaining significantly more weight than control ones.[40] Significant increases in milk production are reported only for primiparous mothers, not multiparous.[41] Milk of treated mothers shows presence of the drug, although the milk:plasma ratio of transfer is lower than with metoclopramide or chlorpromazine. Although side effects are extremely rare in mothers and have never been reported in infants of treated mothers, the AAP advises against use of sulpiride in breastfeeding women as it does with all neurotropic drugs. No information on the use of sulpiride in dogs or cats is available.

Hormones. Oxytocin causes contraction of the myoepithelial cells that surround the alveoli as well as contraction of milk ducts, causing milk ejection. Although available for several decades as a human drug for use during labor or for postpartum hemorrhage, little information is available on its use to increase milk production in women. Earlier studies reported a significant effect of one nasal spray/nostril (3 IU total) of oxytocin prior to manual expression.[42] However, no significant effect on milk volume was observed in a recent double blind randomized controlled trial.[43] A combination of oxytocin nasal spray and metoclopramide has given positive results on milk yield.[44] There is no information on transfer of oxytocin to maternal milk in women, and no side effect has ever been

reported in treated mothers (except perhaps abdominal pain in women recovering from a cesarean section) or in infants. Despite limited scientific information, and despite its well known mechanism of action related to milk let-down, most human gynecologists consider oxytocin capable of increasing milk production and recommend its use when needed.

Oxytocin is widely used in small animals, mostly as a treatment for non-obstructive dystocia due to uterine inertia at doses ranging from 0.5 to 5.0 IU based on body weight. However, most if not all available information on its use during small animal dystocia is anecdotal.[45] No dose-response studies have ever been performed in bitches or queens. Its use in case of agalactia is anecdotally reported as effective following repeated dosing with the nasal spray or with IV or IM administrations of 2-5 IU IM prior to each suckling.[28,37-39,46] Based on its mechanism of action, oxytocin will help to empty the mammary gland of previously produced milk, but it is unlikely to help increasing milk production by the mammary epithelium, unless poor suckling stimulus of puppies is causing failure of milk letdown due to back up pressure on the mammary epithelium. However, anecdotal evidence should not be dismissed as irrelevant as it may sometimes be a prelude to scientific findings; for instance, the anecdotal efficacy of oxytocin in improving milk yield in bitches might be due a potential (unknown) stimulatory effect of oxytocin on PRL secretion. Perhaps oxytocin's greatest impact would be to assist nervous and primiparous bitches with milk letdown in the first hours or days postpartum. The combination of oxytocin in microdoses (0.5 – 1 IU/dog every 60 – 90 minutes, given 15-30 minutes prior to nursing) with dog appeasing pheromone spray (DAP Spray®, Ceva Animal Health, Rutherford, NJ) +/- a low dose of a sedative like acepromazine, can be highly beneficial in causing milk letdown until the bitch settles in with her litter. If a sedative is used, care should be taken to not make the bitch so tired that she cannot care for her litter properly or that she may become clumsy and lay on a puppy and smother it.

Decreasing milk production

Milk production can be easily inhibited in small animal thanks to the availability of antiprolactinic compounds as veterinary (and human) drugs. Conditions for which milk production should be inhibited include pseudopregnancy in bitches, as well as mastitis in lactating bitches and queens. Also, inhibiting milk production should be taken into consideration at weaning, especially if galactostasis (mammary congestion) occurs, in case of eclampsia or for animals being prepared for mammary surgery. Antiprolactinic agents include dopamine agonists and serotonin antagonists.

Dopamine agonists. Both bromocriptine and cabergoline have a strong dopaminergic activity, and thus can reduce prolactin secretion by increasing the action of its natural inhibiting factor, dopamine. Bromocriptine is a non-specific D2-receptor agonist which has been available as a human drug in many countries of the world and used in veterinary medicine since 1980. Several therapeutic protocols have been proposed, with doses ranging from 10 to 100 μg/kg BID for 10–14 days.[48-50] The human formulation (Parlodel™, Sandoz, Princeton, NJ) comes in 2.5 mg tablets, which makes fractioning necessary to achieve the correct dose for bitches, although treating small size dogs can be done by dissolving a single tablet in 10 ml distilled or sterile water in an amber vial, providing an equivalent of 250 mg/ml solution; the solution should be refrigerated and used within one week to maintain its effectiveness. The dose-dependent inhibiting effect of bromocriptine on PRL secretion is strong but relatively short-lived (half-life: ± 4-6 hours). Clinical efficacy in inhibiting mammary function in pseudopregnant bitches is achieved by administering bromocriptine BID at doses of 10-30 μg/kg,[51] although doses as low as 7.5 μg/kg are reportedly effective.[52] Its lack of specificity leads to side effects such as vomiting and anorexia (the most common ones, especially at doses > 30 μg/kg) due to stimulation of the chemoreceptor trigger zone, and less commonly (or with higher dosages) also effects the cardiorespiratory system such as hypotension due to vasodilatation (adrenergic type effect), and/or depression and behavioral changes.[53] In our experience, gastrointestinal side effects can be lessened by giving the medication with food, while attempts to improve side effects by gradually increasing an initially low dose, or by pre-treating with an anti-emetic drug have proved only partially effective. Although its effectiveness has never been questioned, bromocriptine is not approved in most countries as an anti-PRL in small animals and its extra-label use has not caught on, in spite of its world-wide availability as a human drug.

Cabergoline is a synthetic ergot derivative with a long-acting, selective D2-dopamine antagonist effect, and low affinity for dopamine D1-receptors, α1-adrenergic-receptors and α2-

adrenergic-receptors. Following a single oral dose, absorption of cabergoline from the gastrointestinal tract is highly variable, typically occurring within 0.5 to 4 hours. Ingestion with food does not alter its absorption rate[21,54,55] Its PRL-inhibiting action is very effective[21,22,52] and longer than 48 hours due to its particularly long (minimum 48 hours) half-life at the hypophyseal level. This has clinical relevance, as unlike other antiprolactinics requiring twice daily administrations, cabergoline can be given once daily. The most common side effect is vomiting, which appears to have a lower incidence with respect to other antiprolactinic agents due to cabergoline's specific D2-type action as well as to the fact that the drug rarely crosses the blood–brain barrier and consequently has much less central side-effects.[21,22,56] The veterinary formulation comes as oral drops (Galastop™, CEVA) and its suggested dosage in bitches is 5 μg/kg (0.1 ml/10 kg) per day for five days, and in queens of 0.5-1.0 ml/cat. An injectable formulation has recently become available in Italy.[57] When administered at the dose of 1.5 µg/kg injectable cabergoline is effective in reducing the circulating PRL concentration in lactating bitches, with mean PRL values showing an average reduction of 50% with respect to the placebo period three days after the administration, and PRL concentration remaining significantly low for 60 hours post-injection.

Serotonin antagonists. Serotonin is considered the main stimulating factor for PRL release. Therefore, a serotonin antagonist will depress PRL release by depriving pituitary lactotroph cells of a necessary stimulus. Metergoline is a synthetic ergot derivative which functions as a serotonin antagonist, and as such it deprives PRL secreting pituitary cells of a necessary trophic stimulus thereby inhibiting PRL secretion via an indirect mechanism. However, at high doses metergoline also displays dopaminergic effects[50,58] Reported side effects include behavioral changes such as anxiety, aggressiveness, hyperexcitation and whining, which are due to its central antiserotoninergic effect[48,59,60] Because of these central effects, metergoline should not be used in aggressive, anxious or nervous bitches or bitches with behavioral problems, as these may be exhacerbated. Vomiting may occur, but is less common than with bromocriptine. Because of its short serum half-life metergoline's antiprolactinic effect is lower than that of cabergoline and bromocriptine,[61] although the veterinary formulation appears to be satisfactory for use in small animal practice when using the suggested lactation inhibiting dose of 100 µg/kg orally BID, to be continued for eight to10 days. The veterinary formulation (Contralac™, Virbac Animal Health, Ft. Worth, TX) comes in 0.5 mg and 2.0 mg pills.

Clinical considerations

Because of the key role played by PRL in determining mammary growth and milk secretion, antiprolactinic agents constitute a very effective way to decrease milk production, such as in case of pseudopregnancy [22,56,58] Pseudopregnancy is a condition typical of canids which evolves as an adaptation from wild to domesticated life. As such, it should be considered as a physiological phenomenon which may or may not require treatment depending on how it develops. Development of mammary glands occurs normally during diestrus, and unless it is accompanied by milk secretion and nesting behavior it should not be a cause of concern or a reason for treatment. However, owners should be instructed that when milk secretion appears the bitch should be watched closely as this is often a threshold for initiating treatment, especially if accompanied by behavioral signs. Mild cases should be given a few days of close observation before treatment is instituted, as spontaneous remission is not uncommon especially if the bitch does not lick or suckle on herself. Stimuli such as licking, milking and the use of cold and hot packing should be avoided as they will cause oxytocin release (and might be associated with PRL release as well). Whenever licking or maternal behavior are displayed, pharmacological treatment to inhibit PRL secretion and mammary function may be instituted. Antiprolactinics are the drug of choice to stop milk production and eliminate behavioral signs in pseudopregnant bitches. In pseudopregnant bitches behavioral and mammary signs will disappear following treatment with a progestogen; however, a PRL peak frequently occurs when progestogen concentration declines following treatment withdrawal, which will cause the condition to recur. The PRL peak at the end of treatment is to be expected as this is what is observed at the end of a normal luteal phase,[62] or when ovariectomy is performed during a luteal phase.[63] Therefore, progestogens should never be used for treating canine pseudopregnancy.

The length of treatment of most antiprolactinic protocols is five days. However, this duration of treatment is sometimes not sufficient to treat the condition successfully, and relapses are occasionally observed. When trying to inhibit mammary secretion, it is always advisable to treat for

at least 10 days, as this almost eliminate the risk of recurrence. Occasional failures can be dealt with by repeating the treatment course and also by using joint protocols of cabergoline+metergoline or cabergoline+bromocriptine. If an antiemetic becomes necessary (which is rarely the case with cabergoline and metergoline) its choice should be made with caution as many antiemetic drugs may cause PRL release in the dog and so are contraindicated since they are counterproductive. As to treatment choice, in a comparative study over time, changes in plasma prolactin concentrations after one oral administration of bromocriptine (25 μg/kg), metergoline (200 μg/kg) and cabergoline (5 μg/kg) were followed over a period of three days with frequent samplings; an effect of the three drugs was observed after 1.5, 4 and 4 hours, respectively. Maximum effects and interval to return to basal values were: bromocriptine four hours and lasting for ± two hours; metergoline four hours and lasting for two hours; cabergoline six to eight hours and lasting for up to 48 hours.[64] Cabergoline seems to offer some advantages over other PRL-inhibiting substances. However, vomiting does occur occasionally, and sometimes a bitch will vomit with cabergoline and not with metergoline. Both drugs are considered effective, the only precaution being to avoid metergoline in bitches with behavioral problems.

Timing of antiprolactinic treatment has never been debated so far. Prolactin secretion has been reported to be pulsatile perhaps due to stimuli provided by suckling pups.[65] A diurnal variation in serum PRL concentration was observed in three of ten anestrous bitches,[66] with high values between 2:00 pm and midnight and low values from 1:00 am until noon. In a group of adult, lactating Beagle bitches we observed a clear circadian rhythm of PRL secretion, with the highest values found in the morning, and the lowest values found in the evening.[57] These results need further investigation, as there might be differences in the mode or pulsatility of PRL secretion depending on the stage of the reproductive cycle or the condition of the bitch. Pulsatility of PRL secretion might be relevant for timing of antiprolactinic treatment: in fact, if a peak PRL secretion occurred in the morning it might be ideal to plan a once daily treatment starting in the morning rather than in the afternoon or evening.

Conclusions

Controlling mammary function is a topic which has been neglected in the veterinary literature, however it is becoming more common as a presenting complaint in small animal practice. Stimulating mammary growth and milk secretion remains a challenge, although there is a wealth of human drugs which are being increasingly used in bitches and queens. Small animal clinicians need to be aware of these compounds, their indications and contraindications as well as suggested dosage and potential side effects in dogs and cats. The use of antiemetics, particularly of peripheral dopamine antagonists such as domperidone, may help save litters of puppies and kittens.

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A review of mammary gland neoplasia in the bitch and queen

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Abstract

Mammary gland tumors are amongst the most common tumors of bitches and queens. About half of all tumors of the bitch arise from mammary glands and from one third to one half of these are malignant, making mammary tumors the most common malignant neoplasm of the bitch.[1] In the queen, tumors arising from mammary tissues are the third most common occurring neoplasms after skin and lympho-hematopoetic tumors. Nearly all mammary gland tumors in cats are malignant.

Masses involving the ventral abdomen are occasionally not of mammary gland origin. The differential diagnosis includes skin tumors, foreign body reactions, hernias, neoplasms of other tissues, etc. Mastitis should also be considered. Another primary proliferative, but non-neoplastic mammary gland disease that can present as sudden growth of one or more mammary glands in young cats is a benign condition referred to as fibroadenomatous hyperplasia.

The majority of canine and feline mammary gland tumors arise from epithelial cells of either the glands or ducts. These become either benign (adenomas) or malignant (adenocarcinomas or carcinomas) neoplasms. "Mixed" mammary gland tumors are unusual in that they contain both neoplastic epithelial and mesenchymal cells. These occur in the bitch, but rarely in the queen.

Histopathology is the gold standard for tumor identification and characterization. Unfortunately, the nomenclature used by diagnostic pathologists can be complicated. For example, most classification systems include even very rare mammary tumors, which can make them seem overwhelming.

Several different classification schemes have been published that categorize mammary tumors of cats and dogs based on microscopic features. These have proven to very useful for clinicians. Histopathology findings provide important prognostic information and regardless of the classification system used, they divide tumors into those that are likely to behave in a benign manner from those that have features that identify them as malignant "cancers". Additionally histopathology allows for accurate staging of the neoplasm in a given patient.

Features that significantly affect survival of affected bitches and queens include: anaplastic phenotype of the neoplastic cells, tumor size and evidence of metastasis.

Keywords: Mammary, pathology, canine, feline, neoplasia, histopathology

Introduction

The incidence rate for canine mammary cancers is much higher than that for feline mammary cancers (198 and 12.8 per 100,000 in bitches and queens, respectively).[1] Of those surgically removed from the bitch, over 30 percent are malignant. The likelihood that a tumor arising from mammary tissue in a queen will be malignant is much higher and the ratio of malignant to benign feline mammary tumors ranges from four to nine malignant cases for each benign case.[1] Mammary tumors usually occur in females, rarely occur in males, and in the bitch, early ovariectomy confers significant protection against future development of mammary tumors.

Mammary gland anatomy

Mammary tissues respond to hormones. Dramatic changes occur in the gross and microscopic appearance of these tissues during different stages of the estrous cycle (figures 1 and 2). Much of the mass of mammary tissues during the pre-pubertal, anestrual, and geriatric periods is composed of adipose tissue with glandular and ductal tissues being very scant (figure 1).

There are four pairs of mammary glands in the queen, designated T1 and T2 for the thoracic pair and A1 and A2 for the abdominal pair. "Rudimentary" glands are present in the inguinal area in some queens. Lymphatic drainage does not cross the midline and somewhat surprisingly runs in both directions. Lymph, and hence neoplastic emboli that travel via lymphatics, tend to drain cranially from

the thoracic glands to the axillary lymph node centers and caudally from the caudal abdominal glands to the inguinal lymph nodes.[2] It is important to remove draining lymph nodes for histopathology at the time of surgical excision of any suspected mammary neoplasm.

Hyperplastic conditions of mammary glands

Mammary tissues are target tissues for steroid hormones, and as such, undergo hyperplastic and hypertrophic changes during the estrous cycle. Compare the subgross (1X) photographs in figures 1 and 2.

The degree to which changes in glands during various stages of the estrus cyctl can be detected grossly varies between individual animals. The corresponding dramatic histological changes of these target tissues are readily apparent when one compares the images of glands from a Beagle in anestrus with that of hyperplastic tissues from a Beagle in the diestrus. Exuberant mammary gland development can also occur as a paraneoplastic condition if functional tumors of the pituitary or gonads produce stimulatory hormones. In the cat, both males and females can develop a condition called feline mammary fibroepithelial hyperplasia.

Feline mammary fibroepithelial hyperplasia

This benign growth of mammary glands in the cat characteristically occurs in young female cats, with one or individual mammary glands being involved. Enlargement of more than one gland is common. All glands are frequently involved and enlargement of gland pair across the midline also occurs. This is not a pre-neoplastic condition as secondary bacterial infections can lead to mastitis.

Histologically, the enlarged glands are composed of very uniform proliferation of ducts and alveoli and stromal tissues that support them. The uniformity of the proliferation readily differentiates this exuberant proliferation from neoplastic differentiation found in adenomas, adenocarcinomas, sarcomas, or mixed mammary gland tumors. The condition is seen in males and females that have been treated with progesterone or related therapeutic compounds, in young females during their first cycles, during pregnancy and in pseudopregnant females. It can occur in older animals, but uncommonly so.

If the enlarged glands are traumatized, or if the expansion is so large such that it causes impairment to blood flow, the hyperplastic glands and/or overlying skin may undergo necrosis. Such secondary changes can be misdiagnosed as features of aggressive neoplasia.

Mammary gland neoplasia

Because breast cancer is the second most leading cause of death of women in the US, major efforts have been extended to identify appropriate animal models of human mammary neoplasia. There are many similarities between human and animal mammary neoplastic conditions. Considerable attention is being given to mammary tumors of the cat as one of the most similar. Underlying genetics,[3,4] early stage of cellular events associated with malignant transformation,[5] identification of prognostic features[6-8] and therapeutic responses to neoplastic[2] or hyperplastic conditions of mammary tissues[9] have been studied in cats with spontaneously developing mammary diseases. Mammary neoplasms of the dog have less common features to those of women.

In general terms, the most prominent feature of a neoplastic cell is that it has "unregulated cell growth". Some cells that have undergone neoplastic transformation will behave differently. Depending on the nature and degree to which genetic controls are altered, the biological behavior of the transformed cells can lead neoplastic cells to behave in either benign or an aggressive and uncontrolled way.

If cells are less well-differentiated, their phenotype (appearance) will change and features such as rapid replication and altered cellular activity occur. Histopathologists rely on such changes in cell morphology and cellular behavior to characterize, differentiate, name and classify different tumors. Many studies have shown that microscopic features allow pathologists to classify mammary gland tumors into different types, and that tumors in these different categories will behave in a somewhat predictable way enabling them to provide reliable prognoses. Some tumor types also have predictable responses to different types of therapy. Identification of cellular features that do have predictive value has expanded

greatly in recent years and goes beyond interpretation of morphologic features based solely on classic hematoxylin and eosin stained tissue sections. Use of special staining, cell sorting, and other molecular techniques are employed to finely characterize individual tumors, which is becoming increasingly common as therapeutic modalities expand and specific treatment regimes require more detailed characterization of any given tumor's phenotype. Several references are included in the bibliography as examples.[3-5]

Histogenesis of mammary gland neoplasms

Mammary gland tumors arise most commonly from epithelial cells. They are either secretory epithelial cells that line the alveolar glands or from those that line the ductal system. Tumors arising purely from stromal cells, including contractile "strap" cells that cuff the alveolae, are very rare. In the bitch, a combination of epithelial and these myoepithelial cells occur quite commonly and are called mixed or complex mammary gland tumors. They are less likely to be malignant than solid or ductal carcinomas.

Neoplastic transformation of cells of epithelial origin can lead to growth of tumors that behave in a benign growth pattern and are called adenomas. Those that have a more aggressive growth pattern which results in metastases, rapid tumor growth with subsequent implantation and growth at other sites frequently resulting in associated tissue destruction and/or functional compromise are adenocarcinomas.

Pathology of mammary gland neoplasms

Neoplastic conditions are part of a spectrum of changes cells can undergo. In the extreme, cells that have mutations of critical genes die. Those that cause alterations in genes associated with control of cell cycle and growth, those that affect DNA repair mechanisms, those that cause altered tumor suppression, or change the number of receptors on the surface of cell can lead to the transformation of normal cells to neoplastic ones. From a diagnostic perspective, changes in cell proliferation, growth patterns or evidence of altered function frequently affect the morphologic appearance of cells and the gross appearance of tissues they produce.

It is these types of changes that allow histopathologists to identify neoplasms and to further classify them. Cells that have not undergone permanent changes and thus are not truly neoplastic can resemble tumor cells. Cells that have reactive, metaplastic changes or dysplastic can be difficult to differentiate from true neoplastic conditions. In some, but not all tissues, these changes are considered to be "pre-neoplastic", and under certain conditions, progress to development of neoplasms.

One example of the effects of chronic stimulation of reproductive tissues, recognized some time ago, is the contraceptive use of progestagens. The adverse sequella also extends beyond our domestic species. The use of the synthetic progestin melengesterol acetate (MGA) causes similar diseases associated with hyperplastic and neoplastic conditions in zoo canids[10] and felids.[11] In zoo felids, there is a link between MGA administration and clinically aggressive mammary gland carcinomas.[10,11]

Classification of types of mammary tumors of dogs and cats

Based on common usage, the term "cancer" has become synonymous with "malignant". The phrase "benign cancer" is in common usage, yet less correct. In descriptive pathology, when morphologic features for a given tumor are being assessed, diagnostic pathologists frequently add modifiers that reflect the prominent microscopic pattern or other features. For example, these modifiers may include: solid, papillary, tubulo-papillary, cystic, etc. The tissue patterns may reflect the tissue of origin within a gland. This too is commonly included in the morphological assessment. Tumors arising from the ductal system are described as "ductal" or those with a glandular pattern are considered "adeno...". An example of a tumor that has arisen from the epithelial lining of a duct is presented in figure 4. Note how it extends from its attachment to the inner wall of the duct and has grown to nearly fill the duct lumen.

Two photographs taken at the same magnification as those used for figures 1-4 reveal some of the morphologic changes associated with aggressive mammary gland carcinomas that were removed from two different queens (figures 5 and 6). Areas of central necrosis and invasion into the superficial dermis

causing ulceration are illustrated in the case shown in figure 5. Cancers (malignant tumors) that grow rapidly commonly contain areas of necrosis caused by ischemia as the tissue literally outgrows its own blood supply. Contrast these features to the sections from the cat with mammary fibroepithelial hyperplasia shown in figure 3. As shown at higher magnification in figure 5, the cellular pattern is very uniform and highly organized. Now compare this to the photomicrograph prepared at the same magnification of neoplastic cells epithelial cells from the carcinoma shown in figure 6. Several lymphatics have been invaded by the neoplasm (figure8). For those interested, a more extended discussion of mammary gland histopathology is presented in the work of Dr. Misdorf.[1]

It should be noted that primary tumors arising from other cell types, normally found in mammary tissue or the overlying skin, also occur. These may include: hemangiomas, fibromas, mast cell tumors, sebaceous adenomas, etc. Tumors from other sites can metastasize to mammary tissue, but this is uncommon. Specific diagnosis of mammary masses requires histopathology.

Prognostic features

Neoplasms of feline mammary glands are likely to be malignant, metastasize rapidly, and are often fatal. Simple features, such as size of the mass have predictive value. Following removal, the survival time in affected cats is inversely proportional to tumor size with median survival for cats with tumors greater than 3 cm in diameter being 12 months vs. 24 months for cats with tumors less than 3 cm.[8]

The gold standard remains histopathology. Although different classification schemes have been proposed, regardless of which one is used, tumors types will behave differently.

There are two studies that have assessed histological "grade" and subsequent survival in feline mammary carcinomas (FMC). The studies included tumors in 55 and 84 cats, respectively.[6,7] In the first study done in 1998, three histologic features were assessed and scored based on degree of tubular pattern, nuclear and cellar pleomorphism, and mitotic index. It was determined that there was good predictive value based on degree of tumor anaplasia as it correlated with poor prognosis for survival.[6]

The second study, published in 2011 employed the same histologic criteria used to grade human mammary gland neoplasms. The study also concluded that histologic grading can be used as a prognostic factor for FMC.[7] The criteria were similar to those in the first study: degree of glandular differentiation assessed by prominence of tubular formation, nuclear pleomorphism, and mitotic index. Grade was significantly related to tumor size, clinical stage, lymphatic invasion, mitotic index and survival.[7]

A large study of tumor classification and life span outcome in Beagle bitches was published in 1999.[12] The study included full lifetime evaluations of 1,343 Beagles of which 671 were males and 672 females. All mammary nodules detected were removed as were any mammary nodules detected at necropsy. Tumors were classified based on histologic features using a system published by the World Health Organization. Based on preliminary studies, a modification of this classification was developed and used. This system is referred to as the CRHL (Colorado Radiological Health Laboratory) classification.

The complexity associated with assigning tumors to different categories can be a formidable task as addressed by the authors in this classic paper.[12] They compared the WHO and a widely used system of classification referred to as the Moulton system (named after the author of veterinary pathology textbook on tumors in domestic animals published in 1990).[13] In their study, of the 672 Beagle bitches studied throughout their lives, an astounding 71% developed at least one mammary tumor and 61% had more than one during their lives. Only two male dogs developed mammary tumors.

Based on morphologic features using the CRHL system, of the subset of tumors that arose from epithelial cells and that had caused the death of the bitch (n=73), 66% were classified as "ductular carcinomas". These arose from small interlobular or intralobular ductules (as shown in figure 4) compared to 27% that were classified as being "other" types of adenocarcinomas. More metastases were also detected in bitches with carcinomas identified as ductal carcinomas. At the time, the finding that carcinomas in the bitch have such different prognoses, was unexpected.

Diagnostic pathologists in this country still tend to use a classification system described by Dr. Misdorp.[1] Illustrations of the many types of mammary tumors are included in the papers and book

chapters cited in this paper.[1,12,13] For completeness, each of the classification schemes will include types of tumors that are less common or even rare, which can add to the complexity of trying to understand the diagnostic nomenclature.

From the laboratory diagnostician's perspective, critical questions such as what features of the neoplastic tissue, allows one to provide an accurate prognosis that a given tumor will metastasize or, if left untreated, lead to the death of the animal. From a clinical perspective, the most important question is whether tumor is benign or malignant.

The last 10 years have seen the development of many new powerful molecular investigative techniques and these have been employed to generate a great deal of information about genetics, cell receptors and other potential markers in the study of human mammary neoplastic conditions. Investigations using some of these same markers on tumors from dogs[1] and cats[3-5] have been published. As is so common, with time and additional research, many of the diagnostic tests now being run on samples from humans will make their way into the armamentarium of the veterinary profession. But for the time being, analysis of morphologic features by histopathology does provide critical prognostic information.

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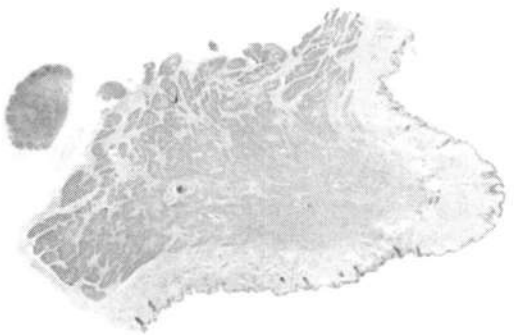
Figures


1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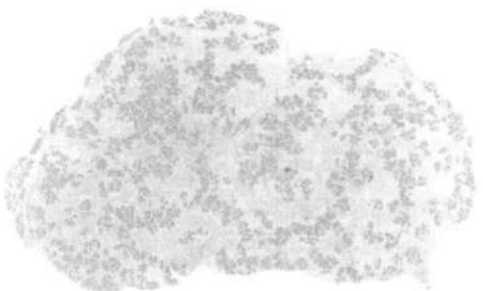
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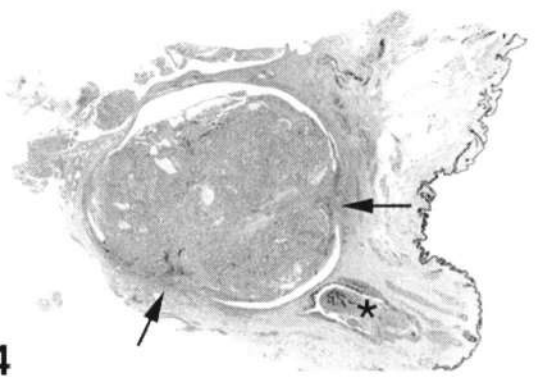

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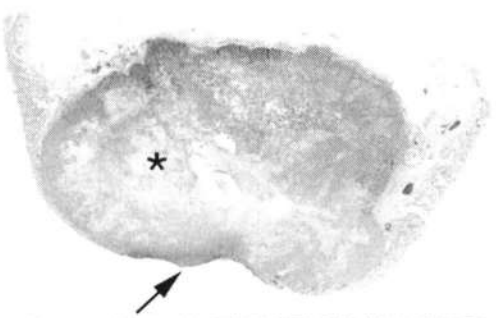

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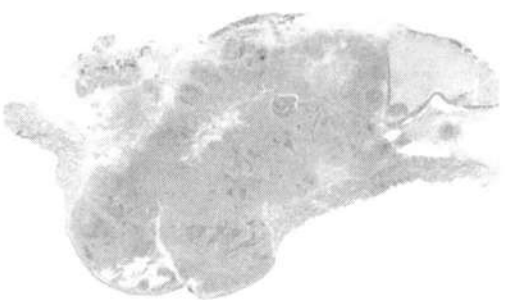
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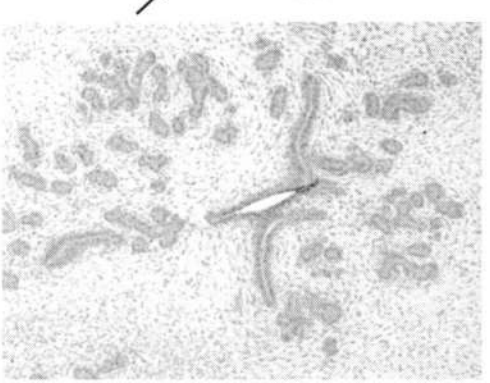
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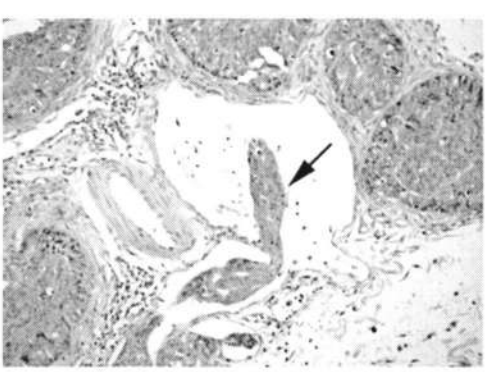
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Figure 1. Normal "inactive" canine mammary gland from a bitch in anestrus. The mammary tissues are inactive. Glands and ducts are dispersed throughout normal adipose tissue, as seen in this photomicrograph as clear spaces above the nipple. Subgross photomicrograph (1X) H and E stain. (Note: Figures 1 through 6 were each taken at the 1X original magnification. The epidermal surface is at the bottom of each of the photomicrographs in figures 1, 2and 4-6.)

Figure 2. Normal hyperplastic canine mammary gland from a bitch during diestrus. The mammary tissues have undergone proliferation. The mammary gland will be enlarged grossly. Mammary tissues at this stage of the cycle are under progesterone influence and are composed of a much greater amount of glandular and ductular structures. Note the uniformity of the tissues compared to neoplastic mammary tissues shown in figures 4, 5 and 6.

Figure 3. Abnormally abundant and uniformly proliferative mammary tissue taken from one of two enlarged mammary glands in a three year old cat. This benign condition is referred to as feline mammary fibroadenomatous hyperplasia. It is a benign condition that can involve one or more glands, tends to occur in young queens, and is not a pre-neoplastic condition. The tissues that undergo hyperplasia and hypertrophy include glandular epithelium, ductal epithelium and myoepithelial stromal cells. These are demonstrated at higher magnification in figure 7.

Figure 4. Papillary ductal adenoma from the mammary gland of a 10 year old Golden Retriever. The large intraluminal mass is attached to the wall of the duct (arrows). A smaller mass is present in the teat sinus (star).

Figure 5. A subgross section of a rapidly growing mammary gland adenocarcinoma in an 11 year old cat. The central area of the mass is necrotic (star) and a circumferential dense rapidly growing rim of neoplastic cells appears as a basophilic band along the expanding and invading peripheral interface with unaffected mammary tissue. The arrow points to an area of epidermal ulceration which is a feature of rapid aggressive growth and, of itself, is evidence of aggressive tumor growth.

Figure 6. This photomicrograph (taken at same 1 X magnification as those in figures 1-5) shows an aggressive malignant adenocarinoma in a three year old Siamese cat. Multiple smaller masses are evident in the adjacent tissues. These neoplastic metastases are shown at higher magnification in figure 8.

Figure 7. Photomicrograph taken from tissues of the enlarged mammary gland in the young cat with fibroepithelial hyperplasia shown in figure 3. Ductal structures are present in the center of the field and very uniformly hyperplastic alveolar glands radiate peripherally. The uniform stromal in which these tissues are embedded is composed of myoepithelial stromal cells. (100 X original)

Figure 8. Photomicrograph taken from the feline mammary carcinoma in image 6 showing invasion of a lymphatic vessel by proliferating neoplastic glandular epithelial cells (100 X original). Several nodules of neoplastic cells that likely represent complete filling and occlusion of other lymphatics are also present.

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

Transcervical endoscopic procedures in the bitch

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Abstract

The use of transcervical endoscopic procedures has revolutionized artificial insemination and diagnosis of infertility in the bitch. Transcervical insemination can be utilized for intrauterine insemination; and is of great benefit during the insemination of chilled or frozen semen and poor quality semen (fresh, chilled or frozen) by improving pregnancy rates compared to vaginal artificial insemination, without the risks of anesthesia and surgery. The endoscope can be used to obtain endometrial culture, cytology and biopsy specimens in the awake, standing bitch, obviating the need for anesthesia and surgery. Endoscopy can also be used for hysteroscopy in the non-pregnant and postpartum bitch to evaluate possible anatomic or pathologic abnormalities of the endometrium. The unique vaginal and cervical anatomy of the bitch requires specialized equipment and training for successful transcervical cannulation, but with practice, clinicians can master this technique and provide useful diagnostics and a unique service to their clientele.

Keywords: Transcervical artificial insemination, culture, cytology, biopsy, hysteroscopy

Introduction

Advanced reproductive techniques in the bitch have increased tremendously over the last 15 – 20 years. The ability to successfully inseminate via intrauterine techniques has increased pregnancy rates and litter size and reduced the need for surgical insemination for intrauterine deposition of sperm. Diagnostic procedures such as endometrial culture, cytology and biopsy as well as hysteroscopy can now all be performed endoscopically rather than surgically. Furthermore, therapeutic procedures such as uterine lavage can now be performed to aid in the treatment of pathologic uterine conditions such as pyometra, mucometra and hydrometra. This paper details the development of these transcervical (TC) procedures, provides detailed descriptions of the techniques, and includes discussion on the various uses of each procedure in a reproductive practice.

Anatomy

A thorough understanding of canine reproductive anatomy is critical to success with TC procedures. The vestibule is short, approximately 2–6 cm long.[1-4] The urethral papilla is located on the ventral floor of the vestibule, immediately caudal to the vestibule-vaginal (v/v) junction.[1-4] The papilla can be both palpated and visualized. Recognizing the location of the urethral orifice is critical, as it is easily entered during the TC procedure and may cause discomfort for the bitch. The v/v junction is a muscular sphincter that normally provides the second barrier of defense for the cranial reproductive tract against advancement of bacteria or foreign bodies (the first barrier is the vulvar lips and the final barrier is the cervix).[1-4] In some bitches strictures or narrowing of the vulvar lips or v/v junction may occur. Additionally, hymenal remnants may be present at the v/v junction (sometimes referred to as 'tags') or vaginal septums may begin at the v/v junction. Digital examination is recommended prior to any TC procedure to determine if any anatomic abnormality is present in the caudal reproductive tract, as they are easily missed when passing the endoscope into the vaginal canal due to the difficulty of insufflating the vestibule. The vagina is very long, extending from 10–30+ cm depending on the size of the bitch.[1-4]

The cranial vagina (also called the paracervical region) begins with the dorsal median fold (DMF), a well-defined fold arising from the vaginal ceiling, that runs from about 3–8 cm posterior to the cervix, leading up to the cervical tubercle.[1-4] The most posterior point of the DMF is called the caudal tubercle.[1-4] There is clear demarcation between the end of the DMF and the cervical tubercle itself and this is an important landmark during TC procedures.[1] There is usually a narrowing in the vaginal canal adjacent to the DMF (particularly in maiden and small breed bitches) requiring gentle pressure to push through.[1-4] There may be discomfort displayed by the bitch in this area, particularly as the endoscope is

moved through it. Once through this narrowing there is usually more room around the cervix itself. The most cranial point of the vagina is called the fornix, and it is a blind ended cul-de-sac.[1-4] The size of the paracervix is important as it may prevent the use of some endoscopes while allowing the use of others; and is the limiting factor in many unsuccessful TC procedures (see below).

The canine cervix sits in a diagonal position at the uterovaginal junction, between 45 and 90 degrees at an upward angle from the vaginal floor.[1-4] The internal os faces almost directly dorsal and the external os faces the floor of the vagina. The appearance of the cervical lumen and caudal tubercle varies greatly from bitch to bitch. In some bitches there are many small furrows giving the appearance of a head of cauliflower, while in others the furrows are fewer in number giving the appearance of the surface of the brain. The author has found that the morphology of the external os of the canine cervix is highly variable. The cervical lumen may be located directly in the center of the tubercle with a clear opening visible or it may be located deeper in the tubercle with a ventral slit and 'ramp' leading to the lumen, which requires manipulation of the cervix with the catheter or endoscope to enter. Because of the steep angle of the cervical lumen and the sharp angle of the uterine body after the cervical canal is traversed, passing a catheter may be difficult even if the cervical lumen can be catheterized. The lumen of the cervix is often narrower in maiden bitches and wider in those that have previously delivered at least one puppy vaginally.[1]

The character of the vaginal epithelium changes with the stage of the cycle.[5] During proestrus the epithelium is pink and edematous. As the bitch progresses through proestrus the mucosa becomes lighter pink and the edema recedes due to dehydration of the vaginal tissues. As the tissues dehydrate, crenulation or wrinkling of the mucosa becomes evident. When the bitch enters estrus, the mucosa appears blanch white due to thickening of the vaginal wall from keratinization and the diminished bloody supply at the lumenal surface. Throughout estrus and the fertile period, the mucosa appears white and crenulated. An abrupt change in the epithelium occurs with onset of diestrus in that the epithelium becomes blotchy red and the wrinkles flatten out. If the mucosa is touched it will blanch and then redden, a phenomenon called rosette formation. As the bitch progresses through diestrus and into anestrus, the mucosa continues to flatten and remains blotchy pink-red. The mucosa in late anestrus is deep pink-red and flat.

Because the colon and bladder both sit dorsal to the reproductive tract, having a full rectum or descending colon or a full bladder may impact the ability to catheterize the cervix. Ensuring the bitch has urinated or defecated prior to attempting catheterization will make the procedure easier. If the bitch will not defecate at the clinic, placing a match (sulfur end in) or a small piece of a cotton applicator swab in the anus and then taking the bitch out to defecate may cause enough rectal irritation to stimulate a bowel movement. The simple act of defecation or urination is sometimes enough to allow passage through the paracervix or to allow presentation of the cervix in such a manner that catheterization is possible.

Types of endoscopes and catheters

Before discussing the types of techniques that can be performed, a brief discussion of the types of endoscopes available and the necessary equipment will be provided. Historically, the first descriptions of transcervical catheterization were with the Norwegian catheter system described Fougner in 1973 and Anderson in 1975.[6,7] The first descriptions involved use of these catheters for intrauterine insemination (IUI). This system involves a hard nylon outer cannula with a metal stylet running through its center. The stylet has a rounded tip to minimize trauma to the reproductive tract during the catheterization procedure. The cannula is initially passed through the vaginal canal to the area of the dorsal median fold and then past the fold as far as possible into the paracervix. Then the stylet is advanced into the fornix. The cervix is grasped through the ventral abdominal wall and manipulated posteriorly to present the external cervical os toward the tip of the stylet. The tip of the stylet is moved systematically back and forth over the surface of the cervix until the lumen is located. There is a typical 'cobblestone feel' of the surface of the cervix and a distinct 'give' once the stylet enters the cervical lumen. Once the lumen is entered, the stylet is advanced proximally a few centimeters and then the bitch is inseminated while the cervix is occluded with digital pressure via the abdominal wall. If the stylet is not in the uterine lumen,

there is significant leakage between the stylet and outer cannula outside the bitch, making it obvious immediately that uterine cannulation has not been successful. This technique requires significant practice to master. It can be performed in most small, medium and large breed dogs. Bitches that are very fat or have deep or tense abdomens are difficult to palpate the cervix, making this technique difficult to impossible. The Norwegian catheter may be used for IUI of fresh; chilled; or frozen semen. It has been used for uterine culture as well, but is not used routinely for this purpose (personal communication, Catharina Linde-Forsberg, 2012). Potential complications include trauma to the vaginal wall, cervix, or uterus and vaginal or uterine perforation.[8]

The first description of endoscopic transcervical catheterization (TCC) was made by Marion Wilson in 1993.[9] She used a cysto-urethroscope for the procedure (Extended Length Cysto-Urethroscope, 27027KL, Karl Storz Imaging, Goleta, CA). The endoscope has a telescope with a 30° oblique viewing angle, an outer sheath, a bridge, and a cold light source. The working length of the endoscope is 29 cm. The system will accept as large as a 9 Fr polypropylene (PP) urinary catheter, although smaller catheters may be used. The diameter of the sheath is 22 Fr (7.3 mm). There is a smaller version of the same endoscope which will accept up to a 6 Fr PP catheter, with an outer dimension of the sheath being 17 Fr (5.7 mm). In smaller or maiden bitches, if the larger endoscope will not fit, the smaller one may allow entrance to the paracervical area. The main disadvantage of the smaller endoscope is that the 5 or 6 Fr catheters are more flexible and so it can be more difficult to catheterize a tighter cervix because the catheter bends rather than threading up the cervical lumen. Minitube® (Minitube of America, Verona, WI) makes TCI catheters that are made of a sturdier plastic and they have a mandrel which makes the catheter more rigid (see below for discussion on catheters).

The endoscope sheath can be fitted with an obturator which can be used to initially feed the sheath through the vaginal canal. This is particularly helpful with smaller bitches as the rounded end of the obturator is less likely to get caught in the vaginal folds particularly in the area of the dorsal median fold where the vaginal canal narrows considerably. A video camera can be attached to the telescope, which greatly facilitates the TC procedure. The 30 ° viewing angle provides excellent visualization of the entire vaginal canal, making this an excellent endoscope for vaginoscopy and vaginal exploration (i.e. for foreign bodies or masses). The bridge has a dorsal port that allows passage of a PP catheter or a small grasping or biopsy forceps beside the camera. There are two side ports on the sheath that allow for insufflation of air (or CO_2) or fluid administration.

Because of the angle of view, and the open sheath, manipulation of the cervix is difficult with this endoscope. If the cervix is not at an appropriate angle, catheterization may not be possible. The inability to get under the cervix with the endoscope to access the lumen may preclude catheterization despite being able to see the location of the external os. Maiden bitches are more difficult to catheterize and may prove impossible to catheterize with this endoscope both due the small size of the paracervix and fornix as well as the lack of stretching of the cervical lumen that occurs following fetal delivery. Small and toy breed bitches may not be able to be catheterized because it is not possible to get the endoscope through the paracervix because there simply is not enough room. Giant breed bitches may not be able to be catheterized because the length of the endoscope is not sufficient to reach the cervix.

Recently, a human ureteroscope has been adapted for TC endoscopic procedures. The ureteroscope (Karl Storz Imaging, 27002L) has a telescope with a 5° end on viewing angle, an instrument port, sealing system and a cold light source. The working length of the ureteroscope is 43 cm, the outer diameter is 9.5 Fr (3.15 mm) and the system will accept up to a 6 Fr catheter. Standard urinary PP catheters will not work with this ureteroscope as they are not long enough. Minitube® makes a TCI catheter (56 cm) that allows 6-10 cm of the catheter to enter the uterus during an insemination procedure. The ureteroscope has a seal for the working channel through which the catheter passes directly through the center of the instrument. There are two side ports which allow either fluid administration or air (or CO_2) insufflation. The seal can be used repeatedly but tends to tear after multiple uses making insufflation less effective as the seal becomes less tight. These adaptors can be replaced but they are expensive, so making them last as many procedures as possible is ideal.

The ureteroscope has some clear benefits over the cysto-urethroscope. First, its small diameter allows it to be passed easily through the paracervical region of the smallest bitches, regardless of their parity. Second, its length allows it to be used in the largest of bitches since the cervix is easily reached. The 5° angle of view allows the ureteroscope to be placed directly in front of the cervical lumen allowing better access to the cervical lumen. In cases where the cervix is angled to the side, downward or upward, the end of the ureteroscope and the catheter can be used to manipulate the cervix into position to allow catheterization of the cervix even in the most difficult of positions. The ureteroscope can be 'walked' up the external os of cervixes with a 'ramp', allowing catheterization of these more difficult cases. The ureteroscope is small enough to be passed through the cervix and into the uterus for hysteroscopy.

Once the handling techniques are mastered, the length of time to perform TC procedures is markedly shorter with this instrument than with the cysto-urethroscope. Uterine culture, cytology and biopsy can be performed using the ureteroscope. Cystoscopy can also be performed with this instrument but visualization is not as good as with the cysto-urethroscope. The disadvantages of this instrument are that the amount of light put out by the telescope is much less than the cysto-urethroscope and the angle of view is also much narrower, so this is not a good instrument with which to perform vaginoscopic examination; and the channel in the ureteroscope is quite small, minimizing the number and size of instruments available for use with it. It is very easy to pass this ureteroscope into the urethra with no signs of distress from the bitch. Due to the small size of the end of the uteteroscope, inadvertent or sudden movement by the bitch may hold a greater risk of vaginal perforation; that said, the small size of any perforation will likely heal quite uneventfully without any surgical intervention.

Minitube® has a TCI shunt which can be used to help keep the vaginal canal insufflated continuously during a TC procedure with the ureteroscope. This shunt is basically a large diameter (36 Fr) Foley catheter. It comes in two lengths (21 cm and 16 cm). The catheter is passed beyond the vestibule and the Foley balloon inflated with up to 100 ml of air. The correct amount of air is usually obvious when the bitch's tail flags as if a copulatory lock has been obtained. The Foley is seated at the v/v junction by pulling caudally against the muscular sphincter there. Then an insufflation hand pump attached to the ureteroscope is used to distend the vagina with air. This air also distends the paracervical area, often lifting the cervix up slightly and making the cervical lumen more accessible. The main advantages of the shunt are that it requires less continuous insufflation by the operator and that it increases the amount of room in the vaginal cavity by keeping it distended with air. When one is learning how to use this instrument, not having to continuously insufflate is helpful. The main disadvantage is that the ureteroscope is less able to be manipulated cranially and caudally because it is 'locked' in the O ring in the center of the shunt. If the bitch moves suddenly, there is less control over the end of the ureteroscope and a greater risk of perforating the vaginal canal. Other disadvantages are that if the bitch has a stricture, hymenal remnant or septum, the shunt may be too large to pass through it so a seal cannot be established; and that the ureteroscope tends to 'stick' to the O ring if the procedure takes more than a few minutes because any lubrication applied to the ureteroscope will dry – this may result in the O ring popping out of the metal holder, an abrupt loss of air from the vagina and possibly frightening the bitch.

Catheter choices include a standard length urinary PP catheter or the Minitube® TCI catheters. When using the cysto-urethroscope either catheter can be used. With the ureteroscope, only the Minitube® catheters will work. Benefits of the PP catheters include the ability to watch the semen (or fluid) enter the uterus and to know the catheter is empty when semen can no longer be seen; the rigidity of the 8 Fr PP catheter when trying to catheterize a highly mobile cervix; the ability to heat mold the catheter at a slight angle facilitating entry into the cervix; and the ability to cut off the end of the catheter and aspirate fluid that is pooling in the fornix when it obscures the view of the cervical lumen. The disadvantages of the PP catheters include the flexibility of the smaller catheters resulting in them bending rather than threading into the cervical lumen, the need to 'burp' the catheter to bleed it of air before threading it all the way into the uterus (this can result in the catheter slipping out of the cervix if the bitch moves suddenly and the need to re-catheterize), the relatively large amount of residual fluid left in the catheter after insemination (up to 0.5 ml in an 8 Fr), and their shorter length such that they can nott be

used with ureteroscope. The PP catheters can be re-sterilized up to three times as long as they do not become bent during the catheterization process.

The TCI catheters made by Minitube® come in 4, 5, 6 and 8 Fr sizes and are either single port or dual port catheters. Fluid flows proximally from the side port openings (up the uterine horn) regardless of single or dual port status. This helps prevent backflow as long as the liquid is injected very slowly and excessive volumes are not used. They are marked every centimeter on the outside of the catheter, so it is easy to determine exactly how far into the uterus the catheter is seated. They are further marked every 5 - 10 cm with a double or triple hash mark to indicate a set length along the catheter. These catheters come with a removable mandrel (a thin metal stylet) that makes the catheter more rigid during the catheterization process. Once the catheter is seated fully in the uterus, the mandrel is removed. Careful attention should be paid to ensure the end of the stylet does not protrude from the side ports; as can happen if the mandrel becomes bent at all during attempts to catheterize the cervix. Once the mandrel is removed a syringe adaptor must be attached. Care should be taken to fully seat the adaptor on the end of the catheter so there is no leakage and the syringe does not fall off. During the learning process, there are many steps needed with these catheters so having an assistant available is helpful. As one becomes more dexterous with the endoscope and catheters, insemination procedures can be performed by a single person. They are dark gray in color so you cannot see the contents of the catheter as they enter the uterus. The 5 Fr catheter has an inner volume of 0.2 ml, so once all the semen is inseminated, a column of air of 0.2 – 0.3 ml should be injected to completely empty the catheter. The smaller catheters have lower inner volumes, so injecting at least 0.2 ml should be adequate for either 3 or 4 Fr catheters. Because of the small luminal size, these catheters empty almost completely, leaving no residual ejaculate in the catheter, assuming air has been injected following the ejaculate. The manufacturer recommends single use for these catheters, but the author routinely cleans and gas sterilizes them (see next section) up to three times as long as they do not become to bent or distorted during a given procedure.

Sterilization of equipment

Following use of the endoscopes, shunts and catheters, they should be rinsed thoroughly in tap water to rinse away any blood, sperm, cells, or bacteria. Both the inside and the outside of the endoscope, catheters and shunts should be rinsed. After rinsing, sterilization of the metal portions of both endoscopes is recommended with glutaraldehyde (Cidex®, New Brunswick, NJ) for 20 minutes. Then the disinfectant is rinsed clean with tap water, followed by distilled water. The metal is dried with canned air - in particular the ureteroscope should not be allowed to air dry because the channel is so narrow that it can take days for it to dry completely and molds may grow on the interior of the channel in the interim. The cysto-urethroscope can be air dried if it will not be used for at least 24 hours – it should be tapped on end several times during this period to encourage any water drops from clinging to the sides. The seal for the ureteroscope can also be placed in glutaraldehyde and rinsed thoroughly. After initial rinsing as described above, the catheters can be rinsed with Alconox® (Alconox Inc, White Plains, NY), followed by ample tap water to remove any soap residue, and then finally with distilled water, prior to drying completely with canned air. The catheters are then gas sterilized with ethylene oxide (ETO) prior to their next use.

Cytobrushes (see endometrial cytology section) are rinsed initially as above, soaked in Alconox® for 5 – 10 minutes, rinsed thoroughly with tap water followed by distilled water and towel dried prior to ETO sterilization. The biopsy forceps (see endometrial biopsy section) are rinsed with water and cleaned with a soft toothbrush to remove all cells and tissue from the jaws. They are sterilized for 20 minutes in glutaraldehyde rinsed in tap water, followed by distilled water and towel dried. Application of instrument lubricant to the jaws will prolong the life of the instrument prior to ETO or steam sterilization. The TCI shunt catheter is rinsed in tap water, soaked in Alconox®, rinsed with tap water and distilled water, air dried for 24 hours or dried with canned air and then either ETO sterilized or steam autoclaved (using pouch mode on the autoclave). The metal portions of the TCI shunt are rinsed and soaked in glutaraldehyde, rinsed with tap and distilled water and then hand or air dried. They do not need to be autoclaved or gas sterilized but they can be if desired.

The author currently uses the cysto-urethroscope for routine vaginoscopy (masses, foreign bodies, searching for the source of vulvar bleeding, etc) and for uterine lavage during the treatment of pyometra. The 30° angle of view and increased amount of light emitted from the larger telescope is highly beneficial for these procedures as well as for cystoscopy. The author currently uses the ureteroscope for all TC inseminations, uterine cultures, cytologies and biopsies as well as for hysteroscopy.

Basic catheterization technique

The bitch should be restrained in a standing position on a table.[1, 8-10] The use of a hydraulic table is quite helpful as the bitch can be raised or lowered to a comfortable position and adjusted as needed during the procedure. Either a wide belt around the abdomen or an assistant can help hold the bitch in position. No pressure should be applied to the abdomen during the procedure as this will obscure the view obtained by the endoscope. Sedation is rarely necessary, particularly when using the ureteroscope because there is virtually no pressure when passing through the paracervix. If sedation is needed, an extremely low dose of either acepromazine, butorphenol or dexmetdetomidine may be administered. Sedation is most likely to be needed when procedures are performed during diestrus or anestrus, when uterine lavage will be performed due to discomfort the bitch may have as the uterus is distended, or during hysteroscopy. Care should be taken not to sedate the bitch so much that she will want to sit down or rest on the abdominal belt or assistant's arm. In the author's experience, almost all bitches tolerate this procedure very well during any stage of the cycle, particularly when using the ureteroscope. Warming the end of the scope with a hot water bottle or rice bag so that the bitch is not startled when the cold metal is introduced into the vaginal canal is also very helpful.

The endoscope is introduced at a steep angle to the vulvar lips (60 - 80° from horizontal) and the tip of the endoscope is introduced at the dorsal commissure of the vulva, and directed dorsally, so that the urethral papilla is avoided. The urethra has a streaky red – white appearance and is easily identified when the endoscope is introduced. If the urethra is entered, the endoscope is removed and reintroduced more dorsally. Once the tip of the endoscope clears the pelvic rim the operator's end of the endoscope is raised to horizontal and insufflation commences. With the cystoscope, minimal insufflation is needed during estrus, while with the ureteroscope, repeated or continuous insufflation is helpful. The lumen of the vaginal canal is located (using the tip of the catheter as a guide) and the endoscope is maneuvered along the vaginal folds to the paracervix. When the DMF is identified, the endoscope is passed along one side of it. If there is not enough room on one side, sometimes backing up and changing sides will allow for easier passage. The more edema that is present, the more difficult the passage, while when there is marked crenulation, passage is easier. If the endoscope is being passed during diestrus or anestrus it is very important to remember that the vaginal wall is much thinner during these stages and so perforation is much more likely to occur with sudden movement or excessive pressure.

Once the endoscope is maneuvered past the DMF, the cervical tubercle is visible suspended from the dorsal wall of the vagina. If the fornix is reached, the endoscope should be pulled caudally 1-2 cm to locate the cervix. Typically, the cervical os is pointing ventrally so the endoscope must be moved underneath the cervix, moving it from one side to the other until the lumen is located. Watching for bloody discharge or for bubbles of air coming from the lumen can sometimes assist in its location. The catheter is lined up with the opening and advanced forward, using the catheter and the endoscope as needed to manipulate the cervical opening into position. If the cervix is pointing sideways, moving the endoscope to the opposite side and pushing anteriorly with the catheter can help reposition the cervix. Do not forget that sending the bitch out to urinate or defecate can radically change the position of the cervix, so if it is difficult to catheterize immediately, sending the bitch out to eliminate may be very helpful. Once the catheter is started in the lumen, rapidly twirling the catheter in one direction or the other can help thread the catheter through the cervix and into the uterus.

If using a PP catheter, as soon as the second port is at the cervical os, 'burping' of the catheter of all air should be performed if a TCI is being performed. If other diagnostic or therapeutic procedures are being performed, burping is not necessary so the catheter can be immediately threaded several centimeters into the uterine lumen. Sometimes the catheter will become 'stuck' at the junction of the

internal os and uterine body or at the body-bifurcation (b/b) junction. If this occurs the catheter should be backed out slightly and re-advanced using a rapid twirling motion. The catheter should be passed as far in the uterus as possible for AI, culture, cytology and lavage, while for biopsy it is stopped once the biopsy instrument is just inside the uterus.

The learning curve is quite steep with TC procedures, but once enough bitches have been inseminated it becomes easier to learn how to manipulate the cervix and catheter for success. Initially there will be many bitches that are not possible to catheterize but with patience and practice, most bitches can be successfully catheterized.[1,8,9,11] The author recommends practicing only on bitches in estrus until competency is high and then adding on additional procedures during early proestrus, diestrus and anestrus when the procedure will be more difficult or associated with higher risks.

The cervix can be catheterized during any stage of the cycle but the procedure is easiest during proestrus and estrus.[10,12,13] This is because the bitch is receptive to the procedure, and the paracervix region is more relaxed making it easier to pass the endoscope through the vagina.[1,9,10] Reported complications include endometritis, mucometra, hematometra, vaginitis and vaginal perforation.[10,14] These complications are more common when procedures are performed during diestrus or anestrus compared to proestrus or estrus. During diestrus and anestrous the vaginal wall is thin and easier to perforate if too much pressure is applied or the bitch moves suddenly.[9,10] Repeated catheterizations may cause infection.[10]

Transcervical insemination

The volume of semen inseminated should be very small – approximately 1 ml for toy breeds, 1.5 - 2 ml for small – medium breeds; 2 – 2.5 ml for large breeds and 2.5 – 3 ml for giant breeds. Larger volumes of fluid will simply backflow out of the uterus into the vagina and should be avoided.[11] The ejaculate should be inseminated slowly (over 2 – 5 minutes) depending on the amount of backflow visualized. When backflow is noted, the insemination process is halted for 15 - 30 seconds and then started again slowly.

Difficulty visualizing clearly may occur due to accumulation of blood in the fornix, obscuring the view of the cervix or from cellular sloughing in last estrus to early diestrus. With the cysto-urethroscope the only way to clear debris off the telescope is to remove it and clean it or to instill sterile saline down the sheath to rinse the cells away. With the ureteroscope it is much easier, as the end of the telescope can simply be tapped against the vaginal mucosa to clear debris off the fiberoptics. Blood pooling in the fornix may be suctioned out using an 8 Fr PP catheter with the end cut off (open ended) and a 12 cc syringe. Just a small amount of fluid, 1 ml or less, can prevent good visualization of the cervical os.

Site of deposition of the ejaculate does not affect the distribution of sperm in the uterus.[15] Sperm were evenly distributed throughout the uterine lumen within three hours of insemination regardless of whether sperm were deposited at the tip of the horn or in the uterine body. Sperm distribution occurs throughout both horns even when insemination only occurs in one horn.[15] Thus the location of the end of TCI catheter should simply be far enough into the uterus that backflow of the ejaculate does not occur during insemination. If there is resistance to injecting the inseminate, the port of the catheter may be pressed against the endometrium; simply twirling or moving the catheter forward or backward a centimeter or two, usually results in re-establishment of flow.[11]

Transcervical insemination can be used for fresh; chilled; and frozen semen.[1,8,9,16-19] Insemination can be performed either once or twice during the fertile period. With high quality semen, a single insemination may result in normal pregnancy rates and litter size.[16,17] Pregnancy rates over 70% can be expected if frozen semen motility is over 40 to 50%.[17-19] Use of multiple breedings will increase the likelihood of having adequate numbers of viable spermatozoa available at the site of fertilization when oocytes become mature and this will result in increased pregnancy rate and litter size.[9,17-20] Intrauterine breeding doses of 200 x 10^6 sperm are associated with pregnancy rates >80% when quality semen is used. Successful pregnancy may be possible with doses as low as 30 – 50 x 10^6 sperm per insemination.[8,9]

Endometrial culture

Cultures are best performed in proestrus and estrus. This allows for culturing very close to the time of breeding for an accurate representation of uterine flora. If they are performed in the first two to three days of proestrus, results will be obtained before it is time to breed, allowing for appropriate use of antibiotics. It is important to differentiate normal flora from pathologic bacteria, and this can be a difficult distinction.

For this procedure, sterile saline will be infused into the uterine lumen and then aspirated out and saved for culture.[10-14] The author uses a 12 ml syringe of saline, a 3-way stopcock, a 6 ml syringe to collect the sample and a dual port Minitube® TCI catheter. The 12 ml syringe is placed in line with the catheter via the stopcock and the 6 ml syringe attached at a 90 degree angle. The catheter is passed as far into the uterus as possible. A small volume of saline is infused at a time (1-3 ml depending on bitch size). The stopcock is turned 'off' to the saline syringe and 'on' to the collecting syringe. Then the catheter is moved slightly forward and backward while an assistant gently aspirates. Excessive negative pressure on the collecting syringe will result in the catheter port aspirating endometrial tissue into the port, preventing collection of fluid. This is usually obvious if the catheter will not move easily forward and back. If no efflux is obtained, an additional 1-3 ml of saline is infused. This is repeated until 2-3 ml of efflux is collected. In some cases, backflow of saline out of the cervix will be visible. Care is taken not to get the catheter ports too close to the internal os of the cervix as fluid from the vagina can be aspirated and this will contaminate the sample (if air is found to be filling the collection syringe, the ports are too close to the cervix).

The efflux fluid is divided into two aliquots. One to two milliliters are centrifuged, similar to a urine sediment, and the supernatant removed. The pellet is re-suspended and a culture swab used to soak up the pellet suspension. This is then placed in transport medium (Amie's without charcoal is ideal) and submitted for aerobic, *Mycoplasma* and *Ureaplasma* cultures. The remaining milliliter is centrifuged similar to a urine sediment and the supernatant is removed and the pellet re-suspended and smeared on a slide and stained with Wright stain or a modified Schorr's trichrome stain (Diff-Quick®) for cytologic assessment (see below).

Aerobic bacteria are commonly isolated from uterine cultures during proestrus and estrus in low numbers and are similar to the organisms found in the same bitch's vaginal canal and cervix.[12,14,21,22] Bacteria should not be present in the uterus in any significant numbers during diestrus or anestrus, while they are commonly found in the vaginal canal during these stages of the cycle.[10,12-14,21,22] Quantification of bacterial numbers is essential for interpretation.[12,14,21] Isolation of multiple organisms or a single organism in low numbers is likely not significant, while isolation of a single (or two) organism in high numbers should be considered potentially pathologic.[12,14,21] Concurrent evaluation of endometrial cytology can aid in determining the significance of a positive culture (see below). Anaerobes, mycoplasma and ureaplasma are not considered normal uterine flora.[12,14,21] Bacterial species found in the uterus are similar to those found in the vagina.[10,12-14,21] It does not appear that the bacteria found in the uterus during the TC culture procedure are transported there on the TC equipment, since the same numbers and types of bacteria were found in the uterus on post mortem examinations, where care was taken to avoid vaginal contamination prior to sample acquisition.[12] Common pathogens include *Escherichia coli, Pasteurella multocida, group G Streptococci, Staphylococcus intermedius*, and *Proteus mirabilis*.

Endometrial cytology

Endometrial cytology can be performed along with cultures in proestrus or estrus or along with biopsy in very early anestrous.[13,14,23,24] If cytology is obtained with cultures, the centrifuged efflux pellet provides an excellent sample. If performed in conjunction with biopsy, a small cytobrush (Olympus, BC-201C-1006, 1050 cm working length, brush diameter 1 mm, Melville, NY) can be used to obtain the samples just prior to biopsy. The cytobrushes are very small and highly flexible, so that if the cervical lumen is not lined up so it can be easily catheterized, manipulation of the cervix with the cytobrush will not be possible making samples unobtainable. Cytobrush samples are immediately rolled onto a glass

slide and stained with Wright stain or Diff Quick® and provide excellent cellularity and cellular detail. There are reports of a higher incidence of endometritis associated with cytobrush sampling or when samples are taken during diestrus.[10,13,24,25] In the author's experience, however, this has not been the case using the 1 mm cytobrush. Repeated sampling reportedly increases the chances of endometritis.[10,13]

Clumps of endometrial cells appear normal in size, shape and number during proestrus, early diestrus and late anestrus, while they occur in lower numbers during estrus and appear degenerate in late diestrus and early-mid anestrus.[13,23,25] These findings are consistent with the regeneration of the epithelium that occurs during anestrus.[13,23] Single endometrial cells are seen in early diestrus and late anestrus.[25] while clumps of endometrial cells are seen in proestrus, estrus, and diestrus and late anestrus.[13,23] Naked nuclei are seen during proestrus and early diestrus.[25] Bitches with cystic endometrial hyperplasia (CEH) had degenerate endometrial cells, single endometrial cells, foamy cytoplasm and rare neutrophils.[25] Altered numbers of single endometrial cells and naked nuclei are a good differentiator of disease.[25]

Polymorphonuclear leukocytes are the most common white blood cell type seen in all stages of the cycle except anestrus, when lymphocytes and macrophages predominate.[13,23,25] Neutrophil numbers are highest during proestrus and estrus or associated with intrauterine infection.[13,23,25] Lymphocytes and plasma cells are seen in large numbers during late anestrus and are also associated with uterine infection and subinvolution of the placental sites.[13,23,25] Eosinophils were noted during proestrus, estrus and in bitches with SIPS.[25] Watts created a leukocyte scoring system which allows one to compare number and types of WBC in a cytology sample to established norms and determine if inflammation is present (Table 1).[23,24] Bacteria were normally seen during proestrus and estrus but not during other stages of the cycle.[13,23,25] The presence of intracellular bacteria is a hallmark of pathologic inflammation.[25]

Table 1. Interpretation of endometrial cytology throughout the estrous cycle

Stage of cycle	Endometrial cell morphology	Types of leukocyte normally present	WBC score
Proestrus	Non- degenerate	Neutrophils	Low numbers
Estrus	Non-degenerate	Neutrophils	Low numbers
Diestrus	Non-degenerate to degenerate	Neutrophils	Low numbers
Early – mid anestrus	Degenerate	Lymphocytes Neutrophils Macrophages	6 3 1
Late anestrus	Non-degenerate	Lymphocytes Macrophages	7 2

Adapted from Watts 1998. White blood cell score is the ratio of each cell type compared to each other.

Interpretation of endometrial cytology is in its infancy, so clear evidence of pathology needs to be documented with additional positive diagnostics (i.e. culture, ultrasonography) and evidence of clinical disease (i.e. discharge, infertility, absorption, abortion).[13,14,23,24] Cervical discharge, cervicitis and vaginitis commonly accompany endometritis.[24] Endometritis was found to be bacterial in origin 70% of the time and non-infectious 30% of the time.[24] Use of computerized cellular morphometric studies allows differentiation of healthy uterine growth, differentiation and involution from uterine pathology.[25]

Endometrial biopsy

Endometrial biopsy can be performed at any stage of the cycle but it is not recommended during diestrus using TC techniques as the risk of taking vaginal flora into the uterus and seeding the biopsy sites with bacteria is very high.[26] Biopsies will yield the most information when they are taken during stages of the cycle when there is endocrine activity resulting in increased glandular function and avoiding stages of endometrial repair (mid–late anestrus). So proestrus, estrus or very early anestrus (while the glandular epithelium is still under the influence of progesterone but after progesterone has dropped to baseline) are

the best stages to take samples transcervically. Bitches sampled during proestrus or estrus should not be bred on that cycle in case unnoticed uterine perforation occurs during the biopsy; which could result in sperm peritonitis at the time of breeding. Biopsy at any stage of the cycle may result in local endometritis which in turn may develop into pyometra, so bitches should be treated with antibiotics prophylactically for seven days after the procedure.[26] There are reports of hematomucometra and endometritis/pyometra following biopsy during proestrus, diestrus and early anestrus.[26]

Biopsy can be performed with the ureteroscope or the cysto-urethroscope but it is more difficult with the cysto-urethroscope.[26] A 65 cm (working length or longer) long oval jaw biopsy forceps (5 Fr) is passed into the cervical lumen via the endoscope. Once the forceps are in the uterus, the jaws are opened and the forceps advanced slowly and gently until slight resistance is felt (author's method). Alternatively, the biopsy forceps may be palpated through the abdominal wall and gentle pressure applied manually as the jaws are closed to ensure tissue is pushed into the jaws.[26] The jaws are closed and the forceps quickly pulled posteriorly to take the sample. The tug of tissue can be felt when it releases from the wall. The forceps are removed and the sample gently teased out of the jaws using saline flush and the tip of a 25 gauge needle.[26] The samples are very small (1-2 mm or less) so care should be taken to avoid too much handling with the needle as this can cause tissue trauma rendering the samples impossible to interpret. Three to four biopsies should be taken at different locations in the uterus. If the forceps are advanced, meet resistance and then there is no resistance, the forceps should be removed–it is possible that the forceps can penetrate the uterine wall and biopsies of mesentery or intestine may be obtained instead of endometrium. Biopsies should be submitted for processing in formalin, for routine staining and interpretation, by a reproductive pathologist familiar with canine uterine histology and pathology. Studies are currently underway to determine if biopsy samples obtained by endoscopic biopsy techniques are comparable to those taken surgically or after ovariohysterectomy. One prior study showed that only 31.1% of samples were in agreement, but this was done early in the development of the biopsy technique, so may not still be true today.[26]

Hysteroscopy

Hysteroscopy is easy with the ureteroscope but is not possible with the cysto-urethroscope in non-postpartum bitches, while the cysto-urethroscope will pass through the post partum cervix up to day 17-23.[10,13] Hysteroscopy may be performed in late proestrus or estrus when the uterine wall is thicker, however there is glandular activity and erythrocyte diapedesis present, so the development of endometritis may be more likely when progesterone rises post-ovulation.[27] During proestrus and estrus, the cervix is edematous and relaxed so passage of the endoscope is often easier (author's experience). Alternatively, hysteroscopy can be performed during anestrus when there will less chance of the development of endometritis after the procedure. Regardless of when the procedure is performed, a week's course of prophylactic antibiotics is indicated.[27]

Early in proestrus, when there is heavy bloody discharge present, visualization is more difficult.[14] In anestrous, bitches are less willing to stand quietly and since the uterine wall is thinner, any sudden movement may increase the risk of perforation of the uterine or vaginal walls. Bitches in late proestrus and estrus, stand quite receptively to hysteroscopy. It is imperative that the bitch stands without moving during the procedure, so bitches that are anxious or nervous or are panting heavily are best sedated or anesthetized to minimize the risk of perforation.[10,13,14] Any bitch being examined during anestrus should be anesthetized to reduce the risk of perforation of the thinner uterine wall. The cervix needs to be sufficiently relaxed to allow passage of the ureteroscope. It is much easier to perform hysteroscopy in multiparous bitches that have whelped vaginally than nulliparous bitches. Depending on cervical anatomy, some bitches may not be able to be examined while others are quite simple and may be inadvertently examined during other TC procedures by inadvertent movement of the bitch or endoscope.

Hysteroscopy is performed by simply following the TCI catheter into the uterus while insufflating slowly but continuously. Having an assistant available to insufflate is best so the operator can concentrate on directing the endoscope up the uterine horns. Overinsufflation may cause discomfort from uterine stretch receptors and so should be stopped momentarily until the bitch is standing quietly again.

Discharge in the uterine lumen may obscure visualization.[27] Infusion of small amount of saline through the side ports can help clear bloody or purulent discharge from the uterine lumen and improve visualization. Alternatively, saline can be continuously infused, instead of air, to distend the uterus. It is critical to be able to visualize the endometrium at all times during the procedure. It is possible to examine both horns in their entirety in some bitches, while in others positioning of the intestinal tract or bladder may prevent traversing the entire length of horn. The size of the bitch may also dictate how much horn can be visualized; in some giant breed bitches the endoscope may not be long enough to reach the tips of both horns. Biopsy of specific lesions can be taken during the hysteroscopic procedure if indicated.[27]

The normal endometrium is homogenously pink in normal bitches.[27] During diestrus, helical folds of endometrium may be present.[27] The endometrium may be irritated during the procedure resulting in development of petechia due to removal of the epithelium in areas where the endoscope touches the uterine wall.[27]

Uterine lavage

Bitches being medically treated for pyometra will benefit from uterine emptying early in the course of treatment. Uterine lavage can be performed with either the cysto-urethroscope or ureteroscope, but is generally easier with the cysto-urethroscope because of the increased field of view, brighter telescope, and ability to use a larger catheter (8Fr rather than 5 Fr or smaller). The cervix is catheterized with the largest catheter possible in a routine manner, using caution when passing the endoscope through the paracervix because the wall is thin during diestrus and perforation is more likely (perforation with pyometra can result in significant peritonitis, so excessive pressure should never be applied). A liter bag of saline or lactated Ringer's solution is attached to the end of the PP catheter and fluids are run in as quickly as possible. If they do not flow freely they can be hooked up to a fluid pump and forcibly infused. Typically, efflux is immediately seen from the cervix, but if it is not, an appropriate amount of fluids should be added so as not to over-distend the uterus. Aspiration through the catheter can be attempted to recover fluid and purulent discharge if it does not flow freely from the cervix. Having an ultrasound close by during the procedure is helpful. The catheter can easily be visualized with the ultrasound and the operator can easily assess how well the uterus is being evacuated or if the uterus is becoming over-distended without adequate emptying. Once the bulk of the purulent discharge is flushed out, the catheter can be removed. Local antibiotics can be instilled prior to removing the catheter if most of the purulent discharge has been emptied. If excessive discharge remains, the antibiotics will likely not be effective in the face of a large amount of organic material. Lavage provides an almost immediate improvement in demeanor in a bitch with a closed pyometra, so should be considered if the bitch is depressed or febrile or if uterine emptying does not begin early in the treatment course.

Conclusion

Transcervical insemination has been used for many years in the bitch with good success. With the advent of better equipment and with more experienced operators, more TCIs and fewer surgical inseminations are being performed, with very high pregnancy rates. Encouraging breeders to use this new technology makes breeding safer and less stressful for the bitch and allows for more than a single breeding thus increasing litter size and pregnancy rates. Furthermore, the use of TC endoscopic procedures has opened up a whole new world of diagnostics for the subfertile bitch. Previously, the only way to obtain such diagnostics was to do surgery and many breeders did not want to be so aggressive with diagnostics to perform them. Interpretation of samples is still in its infancy and work needs to be done comparing biopsy samples obtained via endoscopy to surgical samples to ensure accurate diagnoses are being made. Cultures of large numbers of single organisms are most significant. Cytologic assessment of endometrial pathology is possible and needs to be further compared to biopsy results. Continued research into the uses of and diagnostic results from TC endoscopic procedures are necessary and are occurring.

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Current and proposed research in canine and feline non-surgical sterilization

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Introduction

Non-surgical contraception and sterilization of dogs and cats is a very active field of research. Techniques employed in this research are not those commonly used in small animal theriogenology and instead hail from oncology, molecular genetics, and other fields both in human and veterinary medicine. Much of the work that is being proposed is proprietary at this point and so cannot be discussed in detail. This review is a description of current research methodologies with some examples of specific research goals, to help practitioners and clinical theriogenologists better understand research as it is published. Few specific papers will be cited; the reader is referred to the website of the Alliance for Contraception in Cats and Dogs for proceedings of symposia with detailed information from specific researchers and descriptions of "think tanks" that have been held to address these methodologies and the larger issues of regulatory approval, manufacture, distribution, and marketing of non-surgical sterilants for dogs and cats.[1]

Contraception is reversible control of reproduction and usually is the goal in human medicine. Sterilization is permanent cessation of reproduction and usually is the goal in small animal veterinary medicine. Associated with that complete cessation of fertility is decrease in reproductive physiology and behaviors that we, as a society, have deemed unacceptable in pets, including exudation of serosanguinous vulvar discharge associated with heat in bitches, yowling and lordosis in queens, mounting in stud dogs, and urine spraying in tom cats. Current research is geared toward identification of a sterilant product that is 100% effective at decreasing fertility for the reproductive life of the animal, that is completely safe to that animal and to other species that may encounter the compound, and that can be administered as a single application in the animal's life. Ideally, the product also would be applicable for use in male and female dogs and cats, would have a clear path to regulatory approval in the United States and other countries, would be economical to mass produce and distribute, and would be appealing to shelter officials, veterinarians, and pet owners as an alternative to surgical sterilization.

Keywords: Contraception, sterilization, non-surgical, cytotoxins, immunocontraception, gene silencing

Surgical sterilization

The current standard of practice for sterilization of dogs and cats in the United States is surgical sterilization. Surgical techniques described in the literature for sterilization of females include ovariohysterectomy (OHE) via laparotomy or laparoscopy, and ovariectomy (OE) via laparotomy or transabdominal or natural orifice transluminal laparoscopy.[2-6] The most common technique performed in the United States is ovariohysterectomy via laparatomy. The most common surgical sterilization technique for male dogs and cats is castration.[7] Surgical sterilization is 100% effective in decreasing fertility and also in decreasing most reproductive physiologic events and behaviors that are of concern to pet owners. The reader is referred to current reviews of the literature documenting pros and cons of surgical sterilization.[8-10] Surgical sterilization meets the primary criteria of efficacy, safety, and single application but is expensive and not readily available to help control owned and free-roaming populations in developing countries, where such populations negatively impact animal and human health.

Intratesticular injection

Another direct way to induce sterility is to destroy germ cells without removing the gonads. This is very difficult to achieve in females and will be discussed briefly under immunosterilization. In males, sterilizing agents have been described that are injected directly into the testes, epididymes, or vas deferens. The reader is referred to a good review of the material by Michelle Kutzler.[11] The first product so approved, Neutersol™, was removed from the market. A product using the same compound, Esterisol™, is set to be approved this year. Zinc arginine is injected directly into the testes of puppies, with dose based on testicular size. The manufacturer of Esterisol™ recommends puppies be sedated before intratesticular injection is performed and cautions against extratesticular movement of the compound, which is associated with severe inflammation of scrotal tissue. An inflammatory and fibrous reaction within the testis impairs continuing spermatogenesis but does not completely inhibit testosterone production. Long-term effect on behavior and development of androgen-dependent disease is not clear. Intratesticular injection meets the primary criterion of efficacy regarding fertility. It is safe if administered properly and requires only one application. The drug is expensive and the need for sedation limits its use, especially for free-roaming populations that may require management without veterinary oversight.

More sophisticated methods of sterilization involve targeting of specific tissues or compounds involved in reproduction. The following is a brief review of relevant reproductive physiology.

Reproductive biology

The ovaries contain hundreds of thousands of primordial follicles at birth, each of which consists of a single ovum (queens) or one or two ova (bitches), surrounded by a single layer of flattened somatic cells called pregranulosa cells.[12] The ova in these follicles are arrested in the diplotene stage of Meiosis I and bear connecting segments that permit interaction between the ovum and the surrounding somatic cells. A cohort of follicles is stimulated to begin development with each estrous cycle. Specific signals determining which follicles should begin development and how many follicles should be stimulated with each cycle are unclear but may include stimulatory factors, such as the phosphatidylinositol 3 kinase signaling network, which itself may be stimulated by insulin or growth factors, or cessation of release of inhibitory factors, such as members of the FOXO family of transcription factors or the tumor suppressor gene PTEN.[13,14] Gonadotropin receptors are not expressed in primordial follicles.

Secretion of pituitary gonadotropins and steroid hormones stimulate follicle development and the physical and behavioral manifestations of estrus. Kisspeptins, neuropeptides produced in the hypothalamus, control secretion of gonadotropin releasing hormone (GnRH), perhaps in concert with other neurotransmitters including neurokinin B and dynorphin.[15] Other direct or indirect regulators of GnRH secretion include metabolic signals such as glucose, leptin, and ghrelin.[15] Gonadotropin releasing hormone stimulates pituitary gonadotrophs to produce follicle stimulating hormone (FSH) and luteinizing hormone (LH), which act on the primary and secondary follicles developed from the primordial follicles. As the follicles develop, the granulosa cells surrounding the ovum secrete estrogen, which causes the physical and behavioral signs of proestrus. In dogs, estrogen concentrations fall about nine days after the onset of proestrus; at this time, a surge of LH is released, causing ovulation, and the bitch will stand to be bred. In cats, ovulation does not occur until the queen is bred or otherwise induced to ovulate. Not all of the developing follicles in a given cohort will progress to be pre-ovulatory follicles, also called antral, Graafian, or tertiary follicles. Factors that determine which follicles will develop to this final stage include FSH, anti-Mullerian hormone, activin, inhibin, and insulin-like growth factors.[16,17] The remaining

follicles undergo atresia. At ovulation of the antral follicles, eggs are released from the follicles into the uterine tube, where fertilization occurs. At the time of ovulation, the egg is surrounded by the zona pellucida (ZP) and a layer of cumulus cells.

Spermatozoa are produced in the seminiferous tubules of the testes. The first stage of spermatogenesis is the proliferation phase, during which spermatogonia undergo mitotic division to replenish the stem cell population and produce a number of cells for further development. The second phase is the meiotic phase during which primary and secondary spermatocytes undergo meiosis to produce haploid spermatids. The final phase is the differentiation phase, a morphologic alteration to produce spermatozoa, each of which has a distinct head with an acrosomal cap, a midpiece, and a flagellum. Protamine packaging of the spermatozoal DNA also occurs during this phase, inactivating transcription.[18] Further maturation of spermatozoa, including acquisition of capability for forward motion and ability to bind the outer layer of the ova, takes place as they traverse the epididymis. After ejaculation into the female reproductive tract, spermatozoa undergo capacitation, a complex interaction including loss of decapacitation factors, efflux of cholesterol with subsequent enhanced fluidity of the plasma membrane and transfer of a variety of proteins across the surface, activation of signal transduction pathways within the spermatozoon, and acquisition of hypermotility and ability to bind the ZP. Capacitated spermatozoa bind to the epithelium of the uterine tube until the ova are present; an undefined signal causes their release and permits binding of the spermatozoa to the ZP. A single spermatozoon binds to one of the ZP glycoproteins; this permits cross-linking with other ZP glycoproteins and enacts fusion of proteins on the head of the spermatozoon with a complex of receptors to form a multimeric zona recognition complex.[18] This binding also prevents polyspermy.

Targeted cytotoxins

Targeted toxins work by binding to specific cells associated with reproductive function and destroying those cells only. This is analogous to chemotherapy. For this to work, a purified toxin must be attached to some sort of transport molecule for delivery to a specific target and that transport molecule must bind to the cell of interest and not to non-target cells. One example that has been published is conjugation of pokeweed antiviral protein to GnRH. As GnRH binds to gonadotrophs in the pituitary and is taken up, the toxin is introduced as well and function of those cells inhibited, decreasing release of FSH and LH. Another example is linking of a cytotoxic fragment of exotoxin A from *Pseudomonas aeruginosa* to a ligand that binds the G- protein-coupled receptor for FSH that is expressed specifically in testicular Sertoli cells and ovarian granulosa cells. With binding, cells that express receptors for FSH will be selectively destroyed. Problems lie in specificity of targeting to the cells of interest and ensuring non-reproductive tissues are not accidentally destroyed.[1] For example, FSH receptors are not uncommonly expressed in tissues of the urinary tract in females of some species.

Immunocontraception

Immunocontraception relies on humoral and cell-mediated immune responses against specific proteins or tissues involved in reproduction. Humoral immunity is mediated by antibody production by B-cells exposed to extracellular antigens. Cell-mediated immunity is mediated by cytotoxic T cells, killer T cells, and macrophages, which often are activated by intracellular antigens including virus-infected cells. Antibodies may bind to regions of interest, blocking receptors and subsequent hormone responses such as stimulation of release of GnRH, or directly blocking reproductive events, such as fertilization. Immunosterilization requires cell-mediated destruction of reproductive proteins or tissues. Primary

concerns with immunocontraception are lack of antigenicity of many reproduction-specific proteins or tissues, desire not to block function of those proteins in non-target tissues, and inflammatory response associated with the immune reaction that may damage surrounding tissue.

Because many of the proposed targets are recognized as "self" and therefore do not elicit an immune response, investigators have tried several things to enhance immune response. These include conjugation to other large proteins and packaging of antigen to create a repeat of antigens every 50-100 angstroms, as is a common presentation on bacteria and viruses that stimulate a strong immune response. Many reproductive targets are immunologically privileged because they are sequestered from the immune response early in embryologic development. While these may be antigenic when administered systemically, the tissues may still be protected from the immune response. For example, spermatozoa are protected from the immune system by the blood-testis barrier and neurons producing GnRH are protected by the blood-brain barrier.

Potential immunogens include GnRH, LH, FSH, receptors for those hormones, ZP, and specific proteins associated with germ cells or reproductive organs. Gonadotropin releasing hormone is a small peptide hormone and is highly conserved, raising concerns about inadvertent immunization of non-target species in any compound distributed as an oral bait or otherwise introduced into the environment. Increased antigenicity requires conjugation of GnRH with a larger protein; those that have been employed include tetanus toxoid and keyhole limpet hemocyanin. There have been many studies completed evaluating use of homologous or heterologous ZP proteins for immunocontraception. One example of a specific protein that could be used as an immunocontraceptive agent is a protein called "maternal antigen that embryos require" or MATER, that is expressed solely by oocytes.

Research in immunocontraception has mostly involved ZP or GnRH-based vaccines. Vaccines using porcine ZP in dogs cause erratic estrous cycling and do not consistently prevent pregnancy long-term. Vaccines using recombinant canine ZP proteins conjugated to diphtheria toxin in dogs caused a rise in titers and subsequent inhibition of ovarian follicular development but did not prevent estrous cycling and pregnancy in all cases. Most ZP vaccine studies in dogs were associated with at least short-term infertility in more than 75% of cases but were associated with prolonged proestrus bleeding and estrous behavior and with ovarian cystic disease.[19-21] In cats, vaccines developed using ZP proteins from dogs, cats, mink, and ferrets all were demonstrated to induce a significant, measurable antibody response but did not protect against pregnancy as the antibodies did not bind to the queen's own ZP in vitro.[22,23] It may be that variation in sperm binding sites on the ZP vary enough among species to minimize the effect of antibodies raised against ZP proteins.[23]

Another reported problem with immunocontraceptive vaccines evaluated to date is the adjuvant used. In one study in cats, using Freund's complete adjuvant, seven of 10 cats developed granulomatous reactions at the injection site and in distant tissues including lymph nodes and brain. One of the 10 cats died of a vaccine-associated sarcoma at the injection site, and three of 10 suffered from hypercalcemia and compromised renal function.[24] Granulomatous reactions also have been reported at the injection site in dogs. A commercial ZP vaccine with Freund's adjuvant (SpayVac®) was available from 2002-2005 through a Canadian company. As of this writing, no ZP vaccine is commercially available for use in companion animal species.

In a study in male dogs using GnRH conjugated to tetanus toxoid, rises in antibody titers against the tetanus toxoid but not against the GnRH were demonstrated. A study in bitches using GnRH conjugated to canine distemper virus proteins demonstrated a rise in titers but no inhibition to conception and pregnancy. A recent study in cats using multiple tandem repeats of GnRH conjugated to proteins

from *Pasteurella* sp. showed high titers against GnRH, lack of follicular development, and no estrous cycling or pregnancy for up to 20 months after vaccination.[25] Finally, GnRH conjugated to hemocyanin from the keyhole limpet and adjuvanted with a commercial preparation using *Mycobacterium avium* (AdjuVac®) has been demonstrated to decrease testosterone and sperm count in male dogs and cats; work in bitches and queens is ongoing. A commercial GnRH vaccine using AdjuVac® (GonaCon®) is reported to be undergoing registration for use in hoofstock by the USDA. There are no reports of a commercial vaccine for companion animals as of this writing.

Several different types of vaccines can be used to induce immunocontraception. Protein vaccines are those that have been described, with a purified protein of interest associated with larger proteins and adjuvants to enhance immune response. Another type of vaccine is DNA vaccine, which introduces DNA that encodes the protein of interest into cells where antigen is then produced and available to the immune system. Either system may be incorporated into virus-like particles or other carriers to enhance delivery of antigen and immune response. Virus carriers that have been used include canary pox, which expresses antigen well and cannot reproduce in mammalian tissues, non-replicating adenovirus, lentivirus, and alpha-virus. One group has demonstrated a non-infectious *Salmonella* sp. vector that colonizes lymphoid tissue, expresses associated antigen, and then self-destructs.[1]

True immunosterilization requires complete destruction of specific cell populations required for fertility. Some work suggests irreversible fertility in females after inoculation with ZP proteins destroyed the population of primordial germ cells, including the rabbit doe, bitch, and female monkey.[26-28] This has not been demonstrated consistently in any species, perhaps because the ZP vaccines employed were adulterated with other ovarian proteins inducing significant oophoritis.[29] In women, the number of primordial follicles must fall to less than 1000 before onset of menopause, suggesting a threshold number that must be maintained for estrus cycling to continue.[13] This limit has not been identified in other species.

The final consideration is need for boostering of immunocontraceptives. Research currently underway is looking at packaging of antigenic molecules in some sort of encapsulated form that would gradually degrade and be released as a self-booster.

Gene silencing

Gene silencing most commonly is accomplished with a class of double-stranded RNA molecules. These molecules, when introduced into a cell containing genes with homologous DNA, block transcription and effectively abolish expression of that gene.[30] Specific types of RNA used in this way, small interfering RNAs (siRNAs) and small temporal RNAs (stRNAs), are themselves regulated by the enzyme Dicer. As a group, these are referred to as interfering RNAs (iRNAs). Introduction of iRNAs to cells of interest decreases gene transcription for a variable amount of time. Specificity of the iRNA is vital, to ensure mRNA of a desirable gene is not blocked unintentionally. Similarly, targeting of the iRNA to specific tissues is vital to ensure that production of the protein of interest is abolished as completely as possible but that transcription is not blocked in non-target tissues requiring that gene product.

Because gene silencing does not permanently shut down cell function, the challenge is to create long-term infertility from a transient silencing event. Examples of how this might be achieved include by silencing inhibitory factors that control apoptosis (controlled cell death) or otherwise altering secretion of gene products required for maintenance of the germ cell population. Another question that has not yet been answered is what percentage of active cells must be silenced to effect a change in reproduction. For

example, if a system could be created that silences 50% of kisspeptin secretion, would that alter GnRH secretion or is there a much lower threshold needed, such that a much higher percentage of cells must be silenced to effect change?

Besides iRNA, another group of compounds that have been described for gene silencing are chemically modified oligonuceotides with a nuclease resistant backbone. These bind to DNA and RNA and interrupt transcription and translation. Because these are not broken down by nucleases, they may be able to exert a long-term effect.

One example of gene silencing would be decreased function of neurons secreting kisspeptin or other factors controlling release of GnRH. In this particular example, a second challenge is accessing kisspeptin neurons within the blood brain barrier. Other examples include silencing of genes in the piwi protein family, which are expressed in male germ cells have some function in male fertility, and silencing of the gene for the MSY2, a protein expressed in male and female germ cells and again, somehow supporting fertility. Mice with homozygous knockout of these gene products are infertile, suggesting that long-term silencing could be a method of inducing sterility.

As with immunosterilants and specific cell toxins, delivery of the iRNAs or these other silencing agents only to the appropriate cells is a challenge. Ideas for how to introduce these compounds into cells include packaging iRNAs into particles that bind to the tissue of interest and activate surface receptor mechanisms; antibodies that bind to an siRNA and a target cell, such that the siRNA is taken into the cell by endocytosis; and aptamers, chimeric RNA molecules that bind protein targets on cells, are taken in by endocytosis, and then are activated by Dicer. Viruses also may be used, as described for immunosterilization.

The Red Queen Hypothesis in evolutionary biology states that continuing adaptation is needed in order for a species to maintain its relative fitness amongst the systems with which it is co-evolving. Reproduction is a biologic imperative for animals, and is a complex system with built-in safeguards and fail-safes we have not yet identified. For example, kisspeptin is produced primarily in the arcuate nucleus and anteroventral periventricular area of the hypothalamus but that kisspeptin neurons also are scattered elsewhere in the brain.[15] There is evidence of stem cells permitting follicular renewal in mammalian species.[31] Movement of proteins across the plasma membrane of spermatozoa during capacitation is fluid and involves formation of lipid rafts that permit construction of complex binding platforms to ensure binding of that spermatozoon to the egg in varying conditions.[18] Some crocodile species can alter the environment of the nest to ensure population balance by gender as eggs hatch, and some social insects, like bees, can evaluate available resources and determine how many offspring to produce in a given season. Current research continues to deepen our understanding of animal reproduction to lead us toward the stated goal of complete control of animal reproduction.

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Potential uses of lamellar bodies to assess canine fetal lung development

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Introduction

In 1988, Stuart Dubin used light scattering to study the refraction index of human amniotic fluid as a measure of fetal lung maturity (FLM). His observations led to the determination of lamellar body (LB) number density. Further study also indicated that lamellar bodies are similar in size to platelets.

Surfactant production is a critically timed event in late gestation. Surfactant is composed of roughly 90 percent phospholipids and 10 percent protein, and it is packaged into the layered storage granules known as LBs. The determination of the LB count in the amniotic fluid of the canine preterm fetus or in the amniotic fluid of a newborn or in the amniotic fluid that has pooled in the vaginal canal due to membrane rupture could help in the assessment and management of pregnancy, delivery and supportive care of the neonate.

In humans, neonates born late preterm or at term by elective cesarean before onset of labor are more likely to develop respiratory distress than those born vaginally. All corticosteriods can stimulate the synthesis and release of surfactants into the alveolar spaces. Corticosteroids also reduce neonatal intraventricular hemorrhage. Administration of corticosteroids to women in this risk category is the standard of care to reduce neonatal morbidity by reducing respiratory distress and atelectasis as a result of insufficient pulmonary surfactant.

Several positive factors favor the use of LB count in the management of canine pregnancies. Because LBs are the size of platelets, the platelet channels of many standard hematology analyzers are capable of accurate measurements. This makes the measurement widely available with a low degree of technical difficulty. Further, the test requires a low volume of fluid, it has a rapid turn-around time, and it has a low associated cost.

The Clinical and Laboratory Standards Institute (CLSI) approved the guidelines for LB count in November 2011, found in CLSI document C58-A.

Bitches are, at times, presented with an impending delivery and no accompanying history of progesterone or luteinizing hormone level determinations at the time of breeding. Therefore there is no accurate determination of a whelping date. Lamellar body count may be helpful in the decision to delay the labor, allow a try at natural delivery, or proceed with a cesarean section. Lamellar body count could also have a role in determining the level of medical care required in newborns that are delivered and appear preterm. Decisions regarding antibiotic therapy, oxygen therapy and corticosteroid therapy could be more accurate with LB count data.

The aim of this investigation is to determine if LB count might be useful in clinical practice.

Keywords: Lamellar body count, fetal lung maturity, neonatal management

Clinical usage of GnRH agonists in small animal reproduction: a review

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Abstract

Gonadotropin releasing hormone agonists have a variety of effects on the urogenital system as well as on behavior in dogs and cats. Although their official indications are limited to controlling fertility, libido and aggressiveness in male dogs, current available information indicates that their use is effective also in inducing estrus in females, blocking cyclicity in queens, preventing male cats from roaming and urine marking as well eliminating the typical "tomcat odor" from their urine. The blocking action on cyclicity of bitches is accompanied by side effects (prolonged heat, increased risk of pyometra) which make this use not currently advisable in the dog, particularly in older females. There is evidence that GnRH agonists can be at least be partially effective in treating post-spaying urinary incontinence in the bitch. Other clinical applications which await confirmation include prevention of mammary tumor metastatic disease, treatment of androgen dependent diseases (benign prostatic hypertrophy, perianal gland adenomas) or treatment of azoospermia in dogs. Side effects of GnRH agonists have been reported in humans but not in small animals, perhaps due to their recent use and shorter life expectancy of dogs and cats.

Keywords: Gonadotropin releasing hormone, contraception, estrus induction, urinary incontinence, behavior modification, azoospermia

Introduction

Historically, reproduction control in small animals has been achieved by inducing a negative feedback on the hypothalamic-pituitary-gonadal (HPG) axis through administration of exogenous progesterone (P4), testosterone (T) or their synthetic analogues. This approach still remains valid for some indications, as a correct and judicious use or natural or synthetic progestogens or androgens poses no threat to reproductive and general health. However, if used for too long, at excessively high dosages or in the wrong patient progestogens may cause reproductive and metabolic side effects.[1] Therefore, the clinical use of these compounds, although largely safe from all points of view, requires a great deal of care in evaluating the patient, the stage of the reproductive cycle as well as all the potential contraindications for each single case. The possibility to block the HPG axis without introducing steroid hormones into the organism would therefore be very advantageous as it would carry fewer risks for the animal and be cheaper for clients.

Despite their vital role for reproduction, endogenous sex steroids may have negative effects on fertility and general health. Cyclical stimulation of mammary glands or endometrium with endogenous estrogens and progesterone is known to predispose the female to develop conditions such as mammary tumors and endometritis/pyometra. Androgens are known to predispose male dogs to prostatic hypertrophy as well as contribute to the growth of prostatic carcinoma and perianal gland tumors. Therefore, gonadectomy has always been advocated as a means to avoid the risk of developing uterine or mammary diseases in females and prostatic or perianal diseases in males. However, both spaying and castration are irreversible modifications which in some countries are considered not acceptable on cultural or psychological grounds. Also, surgical neutering carries the risk of increased incidence of health problems such as urinary incontinence, obesity, change of temperament and dermatological problems.[2]

A recent development in the field of control of the reproductive cycle in carnivores is the use of gonadotropin-releasing hormone (GnRH) and especially its long acting agonists. Gonadorelin is a synthetic form of GnRH, while compounds such as buserelin, deslorelin, goserelin, triptorelin, leuprorelin and nafarelin are synthetic analogues which are available as human as well as veterinary compounds. For instance, leuprorelin (also known as leuprolide acetate), triptorelin and goserelin are almost exclusively available as human drugs, nafarelin is currently studied for its potential use(s) in controlling reproduction in small animals while deslorelin is already marketed for veterinary use in most western countries as a 2.1 mg, 4.7 mg and 9.4 mg implant; the 2.1 mg implant is marketed for use in horses (Ovuplant™), but its extra-label use in dogs is rather common, while the 4.7 (Suprelorin™) and 9.4 (Suprelorin 12™) mg implants are currently marketed in Europe and Oceania

for the control of fertility and aggressiveness in male dogs but their extra-label use in cats is currently being evaluated. This paper will review current and potential clinical applications of GnRH agonists in small animal reproduction.

Potential clinical applications of GnRH agonists

Gonadotrpin releasing hormone agonists act by initially over-stimulating and subsequently downregulating GnRH receptors at the gonadotropes in the pituitary, thereby suppressing the function of the HPG axis. Such a suppressing action on the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) leads to an arrest of secretion of gonadal steroids as well as their by-products. Such blockade of steroidogenesis can be used in dogs and cats for a variety of indications in prepuberal and adult animals. Some clinical applications have already been tested and demonstrated useful and effective, while others are just the result of work in progress or have not been put into practice yet but are theoretically feasible based on the results of experimental studies.

Indications for prepuberal animals

Postponement of puberty

Subcutaneous administration of an early GnRH agonist to prepubertal male and female dogs daily for 23 months partially or completely suppressed dehydroepiandrosterone (DHEA), andostenedione, T, dihydrotestosterone, 5-ά androstanes and estrogens in males, and DHEA and estrogens in females.[3] Treatment caused a reduction in testicular and prostatic volume, absence of secondary follicles in the ovary and atrophy of pituitary LH-secreting cells in both sexes. After a recovery period of 14 months both male and female dogs showed puberty and their fertility was normal thereafter.[3] Azagly-nafarelin at the dose of 18 mg in a single depot device was administered subcutaneously to 10 prepuberal beagle bitches and left in situ for one year. None of the bitches came into heat or ovulated while the 10 control bitches came into heat regularly during the study period.[4] There was no difference in body weight and growth rate between treated and control animals, and puberty in treated bitches resumed randomly and in a non-synchronized manner after the device was removed.[4]

Gonadotropin releasing hormone agonists can be used to postpone puberty in cats. We administered a 4.7 mg deslorelin implant to 12 domestic shorthair European cats, nine females and three males of 1.4-3.1 kg body weight and 3-9 months of age.[5] None of the cats had shown signs of puberty prior to the start of the study, and penile spikes were not present in any of the three male cats. Cats were given a GnRH stimulation test (two blood samplings before and one hr after IV of 50 μg gonadorelin) prior to study onset, and blood samples for steroid hormone assay were collected monthly for 24 months. Three of nine queens showed signs of estrus one week following implantation, but estrus signs gradually subdued and did not appear again until the end of the study. Serum T increased in the three tomcats on the post-GnRH sample to adult levels, but penile spikes never appeared and none of the three toms ever showed postpuberal behavior until the end of the study.[5] Similar results in prepuberal queens were obtained when comparing the effect of the 4.7 mg deslorelin implant in 15 treated and 15 control 4-month old queens followed for a maximum of 18 months with physical examinations and vaginal cytology.[6] Average age at puberty was 281±21 and 178±11 days in treated and control queens, respectively, while there was no difference in weight at the end of the study. One treated queen showed clinical and ultrasonographical signs of pyometra 92 days after implantation and was immediately spayed.

Gonadotropin releasing hormone agonists are evidently capable of suppressing the hypothalamic-pituitary-gonadal axis of prepuberal dogs and cats leading to postponement of puberty for a prolonged period of time. In dogs, use of deslorelin in young animals shows an age-dependent response, with pups of four months showing no estrus following implantation while all pups implanted after seven months of age will show puberty within a short time. Only prepubertal administration is capable of avoiding implantation-induced estrus response.[7] However, detailed information on onset of susceptibility to exogenous GnRH around the time of puberty is not available for dogs and cats. We have observed vaginal cheratinization and a rise in testosterone in prepubertal queens and tomcats, respectively, following administration of a 4.7 mg deslorelin implant; however, these signs were not followed by puberty, which was instead delayed by the implant.[5] Use of GnRH

agonists can be considered as a safe method to postpone puberty in dogs and queens, while more data are necessary in tomcats to draw the same conclusion (although a similar effect is likely to occur).

Indications for adult females

Contraception

The gonadal block consequent to the suppression of the HPG axis achieved with a GnRH agonist causes onset of anestrus in the bitch. An implant of goserelin acetate (Goserelin acetate, Zeneca, Milan, Italy), administered SC at the dose of 60 μg/kg every 21 days for 12 months suppressed cyclicity in nine adult bitches reducing circulating levels of estradiol and P4.[8] Treatment of adult bitches with 3, 6 or 12 mg deslorelin (Suprelorin™, Peptech Animal Health, Australia) suppressed heat for periods varying between 10 (3.0 mg dose) and 20 (6 or 12 mg doses) months.[9] Administration of deslorelin during anestrus or in the early stages of proestrus will inevitably be followed by induction of estrus within 4-8 days after implantation,[9,10] while administration in diestrus is not generally followed by heat induction.[10] However, although a serum P4 concentration of 5 ng/ml is reported as a potential threshold above which estrus is not induced,[9] Fontaine et al. have observed estrus induction in four of 28 bitches treated in diestrus.[11] When a GnRH agonist is administered to an anestrous bitch the initial response of the HPG axis is a strong secretion of FSH and LH, followed by estrus, ovulation and development of corpora lutea. Therefore, if breeding occurs during such an induced phase conception will follow, but the ensuing pregnancy will only progress until shortly after day 30 because of the downregulation of gonadotropins leading to luteal failure.[10,12] Normal pregnancy followed by parturition may occur if a bitch is administered a GnRH implant during the second half of gestation, as it may take up to four weeks to cause downregulation, thereby leaving enough time for normal whelping of live fetuses to occur. The 9.4 mg deslorelin implant has been used in a few bitches, with interval treatment-return to heat of 11 months.[10]

In order to avoid inducing estrus following treatment with deslorelin, Wright et al treated anestrous bitches with daily injections of megestrol acetate at 1.0 or 2.0 mg/kg for 2-3 weeks prior to placement of the implant;[12] four of five bitches treated with 1.0 mg/kg megestrol showed heat, while none of 10 bitches treated with 2.0 mg/kg showed heat with duration of suppression varying among individuals.[12] When the same 2.0 mg/kg dosage of megestrol was administered orally for eight days starting four days prior to placement of a 10 mg deslorelin implant, only one of 10 bitches showed a post-estrus heat response while four of 10 bitches presented a mild vulvar enlargement.[13]

In wild carnivores, deslorelin has been used effectively as a contraceptive at doses ranging from 3 mg (foxes) up to 12 mg (lionesses) indicating that dosing in larger individuals should probably be related to body surface area rather than body weight.[14] Treatment blocked estrus behaviur in the majority of animals leading to lack of conception even when females were housed continuously with males. Simultaneous administration of norgestomet or proligestone in one lioness and three female wild dogs did not suppress deslorelin-induced estrus. Return to estrus in some wild dogs and lionesses was observed to occur 12-18 months after administration. No adverse effects were observed on social behavior, general health and body weight.[14] As secondary sex characteristics tend to disappear following prolonged androgen deprivation, GnRH agonists should be used with caution in male lions as their mane (a feature considered as very important for male lions in most commercial zoos) might disappear.

When administered to adult queens at the dose of 4.7 or 6.0 mg, an implant of deslorelin initially stimulates in most queens follicular growth and estradiol secretion, after which no further evidence of estrus is observed for periods of 4-14 or up to 18-26 months.[15,16] In Munson's study five of 10 treated queens had small estrogen increases after 7.5-14 months at which time they were administered a second deslorelin implant,[15] while in our study we did not show any such increase.[16] It is not known whether deslorelin-treated queens may ovulate if bred, what is their incidence of spontaneous ovulation and what, if any, is their incidence of premature luteal failure as reported for the bitch (see over). General health and social behavior remained unchanged throughout the study period, and introduction of a male did not reverse the deslorelin-induced cycle suppression.[16]

Estrus induction

Administration of deslorelin in anestrous bitches at the dose of 1.05 mg, 2.1 mg or 4.7 mg will induce resumption of cyclicity within 2-9 days. [10,17-21] Interval from onset of proestrus until ovulation and onset of cytological diestrus may be shorter in bitches induced with deslorelin than in spontaneous cycles.[19-21] Leaving the implant in situ exposes treated bitches to the risk of pregnancy loss occurring around mid-gestation due to premature arrest of luteal function.[19-21] Administration of 150 IU human chorionic gonadotropin (hCG) at day 42 after the LH peak does not solve this problem as after an initial stimulatory effect on serum P4 a drastic decline is observed over the next few days.[22] When using a 1.05 mg implant (half of an Ovuplant™) premature luteal failure is less likely to occur but some suppression of luteal function is still observed.[22] Incidence of suppression of luteal function can be diminished (or its effects attenuated) by early removal of the deslorelin implant, provided that it is placed in easily accessible places such as the vestibular mucosa, the medial side of the leg or the post-umbilical region.[20,21,23] Implant removal can be performed either as soon as a vulvar discharge is observed,[23] at the time of the LH peak[20] or at ovulation.[21] Although comparative evaluations of different removal times have not been performed, if one considers reproductive parameters of various studies no clear advantage has been identified in this respect. In bitches induced to cycle with deslorelin ovulation rate,* conception rate,† pregnancy rate‡ and rate of premature luteal regression§ were studied by:

a) comparing treated and control bitches using a 1.05 mg (half of an Ovuplant™) or the entire 2.1 mg implant administered into the vestibular mucosa (VM).[19] Ovulation rate was not calculated, all other parameters did not differ.
b) comparing the VM vs the SC (between the shoulder blades) administration using the 2.1 mg implant.[20] Conception rate was either equal or significantly better and a clear trend for a better pregnancy rate and a lower rate of premature luteal regression were evident for VM (66.7% and 16.7%, respectively) vs SC bitches (37.5% and 37.5%, respectively). A control, non-treated group was not used for this study.
c) comparing treated and control bitches using the 4.7 mg implant.[23] Ovulation and pregnancy rates were similar to controls; conception rate was not investigated; all bitches underwent ovariohysterectomy at day 9-19 post-ovulation, therefore it was not possible to assess occurrence and rate of premature luteal failure.
d) comparing bitches treated in early vs late anestrus using the 4.7 mg implant.[21] Ovulation and pregnancy rates were significantly better for bitches treated in late anestrus. Luteal failure was diagnosed in three bitches, and the only bitch whose owner did not agree to a supporting P4 treatment aborted on day 58 after ovulation. A control, non-treated group was not used for this study.[21]

Deslorelin is certainly an effective drug for estrus induction in bitches; in the studies of Fontaine et al, bitches treated in late anestrus showed heat within 4.2+1.4 days in 97% of cases, ovulation occurred in 83% of cases and quite constantly 12±3 days after treatment, and pregnancy rate was approximately 70%.[11,21] However, prolonged pituitary downregulation causing luteal failure despite early removal or using half dosing remains an unresolved issue. Likewise, prolonged heats and anovulatory cycles have been observed.[10,11,19,21,24] As the interval between implant insertion and ovulation is generally short, it has been suggested to remove the implant no later than 15 days post-treatment (even if the bitch has not ovulated yet) in order to avoid unnecessary ovarian stimulation.[21]

Prevention of mammary tumor metastatic disease

The role of gonadal steroids in the development of mammary tumors is well established. Neoplastic transformation of normal cells is thought to be effected by an "initiator", after which

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* Ovulation rate: *n* of bitches ovulating divided by the total *n* of bitches
† Conception rate: *n* of fetuses divided by the *n* of corpora lutea
‡ Pregnancy rate: *n* of pregnant bitches divided by the total *n* of bitches
§ Rate of premature luteal regression: *n* of bitches in which serum P4 drops to basal levels during pregnancy divided by the total *n* of bitches

abnormal growth is stimulated by a "promoter". The mitogenic action of estrogens on canine mammary epithelium has been described.[25,26] Estrogens are considered potential initiators of neoplastic growth in different species, often in conjunction with other hormones. For instance, the "initiating" role is played by estrogens and prolactin in the rat and mouse, by estrogen plus a placental factor and perhaps a novel pituitary hormone in monkeys and humans. In the bitch, the action of gonadal steroids, especially P4, can create a highly proliferative environment in which an important initiating role is probably played by growth hormone (GH).[27] Under the influence of endogenous or exogenous P4, GH can be secreted by the canine pituitary, and if the progestational stimulus is prolonged GH can be secreted by the mammary gland as well. Therefore, gonadal steroids can have a direct as well as an indirect stimulatory action on the canine mammary gland through the production of their needed co-factor, GH.

Normal and neoplastic mammary tissue features receptors for estrogen, P4, epidermal growth factors and prolactin. The amount of such hormonal receptors decreases proportionately to the increase in the degree of differentiation of neoplastic mammary tissue, with malignant mammary tumors having less hormonal receptors than benign mammary tumors.[28] The use of GnRH agonists has proven effective both in rats with hormone-dependent dimethylbenzanthracene-induced mammary tumors as well as in pre-menopausal women suffering from advanced breast cancer.[29,30] A recent study looked at the effect of goserelin in bitches with spontaneous hormone-dependent mammary neoplasia.[8] Following assessment of presence of estrogen/P4 receptors on a biopsy of mammary tissue, 18 bitches with hormone-dependent lobular/invasive mammary carcinoma were selected and assigned to a control (no drug) or treated (goserelin) group. The nine treated bitches received an implant of goserelin acetate (Goserelin acetate, Zeneca, Milan, Italy), administered SC at the dose of 60 μg/kg every 21 days for 12 months. Goserelin treatment reduced circulating levels of estradiol and P4 and reduced the size of mammary tumors after three months in all treated bitches, with 88% of them showing a relapse-free survival time of two years.[8] Although these results await confirmation, the use of GnRH agonists for the treatment of canine mammary tumors looks promising provided that clinical cases can be selected on the basis of tumor type and presence of steroid receptors.

Treatment of post-spaying urinary incontinence

The involuntary loss of urine which may occur following spaying in the bitch has a multifactorial origin, as demonstrated by the fact that no single treatment (whether medical or surgical) achieves 100% efficacy. The chronic gonadotropin elevation which inevitably occurs after neutering (because of absence of gonadal steroid negative feedback) has been considered as a potential cause of urinary incontinence in castrated bitches. The hypothesis that a downregulation of gonadotropins using GnRH agonists may improve or fully cure urinary incontinence was initially considered valid following clinical trials in which ovariectomized bitches refractory to the use of α-agonists were successfully treated with a GnRH agonist (leuprorelin, buserelin, triptorelin or deslorelin).[31] Because of the initial increase in FSH-LH release, some protocols included also administration of α-agonist treatment for the first three weeks.[32] Results have been quite positive with periods of continence varying from one to some months following a single treatment with different GnRH agonists.[33] Although chronic administration of a GnRH analogue decreases plasma LH and FSH to basal values, in incontinent bitches there is little if any correlation between the effect on gonadotropin levels and the response to treatment.[34] Serum concentration of gonadotropins appears to be involved, directly or indirectly with the pathophysiology of canine post-spaying urinary incontinence, with a greater role played by FSH. However, the exact pathophysiology of this condition has not been clarified yet.[35,36]

Administration of deslorelin has no significant effect on urodynamic parameters, even when bitches respond positively to the treatment, but rather seems to modulate bladder function allowing for a larger bladder filling volume at the same bladder pressure.[32,36] Current clinical information suggests that when treated with a 4.7 mg deslorelin implant, previously incontinent bitches will show approximately a 50% recovery rate, with another 10-20% of bitches showing incomplete response characterized by a reduction in the frequency of incontinence episodes and/or an improvement in the response to pharmacological treatment with α-agonists or estriol.[33] We have also used the 9.4 mg implant in a few cases, with periods of continence being prolonged up to almost a year (data not shown). In a pilot study that we conducted in Brazil on efficacy of a 4.7 mg deslorelin implant for

treating canine post-spaying urinary incontinence, three of six incontinent bitches were fully continent for six months following treatment, and a considerable improvement (continence of one to three months) was observed in the other three bitches (data not shown).

Indications for adult males

Contraception

In male dogs, the use of a GnRH agonist will cause a reversible blockade of fertility. Early studies showed that a controlled-release microsphere formulation providing a daily release of 100-200 mg of a LH-RH agonist causes a temporary increase in plasma T concentration during the first few days (from 1.5 to 43.5 ng/ml) followed by a decrease to castration levels for a prolonged time.[37] Similar results were later obtained with implants of 6.6 mg buserelin,[38] 18.5 mg azagly-nafarelin,[39] or 4.7 mg deslorelin.[40,41] Dogs treated with implants of 6.0 mg deslorelin typically show initially an acute increase in concentration of LH and T, with both hormones becoming undetectable after about two weeks;[42] histologically, disruption of seminiferous tubules and epithelial atrophy are evident as early as day 16 and 41, respectively;[43] clinically, they start becoming infertile within a six week period and resume normal fertility only after several months.[38] A chronic treatment with a 4.7 mg deslorelin implant causes a progressive loss of pituitary responsiveness to GnRH over a period of four weeks with a lack of response to stimulation of the HPG with GnRH or LH already evident at three weeks and being complete at 40 days after implantation.[41] The decrease in testicular size may vary from a 20-30% reduction during the first few months up to 50-60% at 6 months post-treatment.[38-40]

In male dogs treated with a deslorelin implant, complete sterility is thought to occur within a two month period.[38] We recently looked at semen quality in six adult dogs treated with a 4.7 mg deslorelin implant.[44] Complete sterility (based on presence of <10 million of progressively motile sperms (PMS) and semen volume <0.5 cc) was achieved between 23 and 84 days post-treatment, with two dogs being still fertile around 55-60 days post-treatment and beyond. Also, semen motility and total count actually improved during the first month post-treatment, while semen morphology was unaffected throughout the study, although all dogs eventually became aspermic.[43] As libido might increase during the first few weeks post-treatment, clients should be advised about the initial improvement in fertility parameters as well as of the time needed for deslorelin to achieve complete efficacy. Once the implant finishes its action or is removed, testicular size starts to increase after a few weeks, T concentrations return back to normal in about seven to eight weeks and testicular volume is back to normal in about six months.[39] Considering the normal canine spermatogenic cycle of approximately nine weeks duration, male dogs treated with deslorelin will likely prolong their temporary sterile phase for as long as nine weeks on top of whatever is the period of recovery, depending on their testicular conditions at the end of deslorelin treatment.

In adult tomcats the use of a 4.7 mg deslorelin implant stops roaming, eliminates the classical tomcat urine odor, eliminates penile spikes and reversibly blocks fertility;[16] such effects are generally observed in most cats by the end of the second month post-treatment and in the 15 cats of our study lasted 15±3 months.[16] We observed a partial failure of efficacy in one of 15 cats, in which basal testosterone decreased to basal levels but there was still a normal response to periodic GnRH stimulation tests.[16] Another client-owned cat (not included in the above study) treated with a 4.7 mg deslorelin implant became more affectionate with his owner but remained aggressive with other cats and kept breeding queens, one of which gave birth to a normal litter when mated four months after treatment. In tomcats deslorelin acts rapidly and is highly efficacious, but may be characterized by a failure rate of <10% which needs to be investigated further.

Reduction of aggressiveness and libido

The decrease in serum T concentration which follows downregulation of the HPG axis will cause individual animals to become less aggressive. We observed a decrease in aggressiveness based on subjective assessment of number and degree of cage fights while studying serum T secretion in shelter dogs treated with a 4.7 mg deslorelin implant.[40] In our clinical experience, the 4.7 mg deslorelin implant is effective is reducing libido and aggressiveness in male dogs, although occasional failures are encountered; these are probably due to the fact both these aspects of a dog's temperament

are not fully dependant on serum T concentration, but there is also a role of experience in their development.

Treatment of androgen-dependent diseases

Benign prostatic hyperplasia. Benign prostatic hyperplasia (BPH) is the most common canine prostatic disorder, with more than 50% of intact dogs developing histologic evidence of BPH after five years of age. Hyperplasia is probably due to an altered androgen:estrogen ratio, and requires the presence of the testes to start and continue to develop. Dihydrotestosterone (DHT) within the prostate gland probably serves as the main hormonal mediator for hyperplasia. Castration is commonly considered the best treatment as the drastic decrease in androgen secretion causes a 70% decrease in prostatic size (due to atrophy) within nine weeks.[45,46] Following administration of a GnRH agonist, prostatic size decreases in parallel with the decrease of T.[37-39,47] When five adult dogs were implanted with deslorelin at a dose of 0.5-1.0 mg/kg body weight, their prostatic volume decreased more than 50% from week six through week 44, and serum T concentrations decreased 90% from week eight through 32 of treatment when compared to controls.[47] Similar results on prostatic growth were observed following treatment with a 6.6 mg buserelin implant, with disappearance of prostatic cyst and prostate returning to approximate pre-implantation volume by week 48.[38] We have observed disappearance of conspicuous (>17 mm diameter) prostatic cysts following treatment with a 4.7 mg deslorelin implant as well as of larger (20x25 mm) prostatic cysts in six adult male dogs with clinical signs of BPH with a 4.7 mg deslorelin acetate administered every six months;[48] an improvement of the clinical situation of all treated dogs was observed without any additional pharmacological treatment already at the first follow-up visit (one month after implant administration) and no further sign of prostatic disease has been observed subsequently in all dogs without any other type of pharmacological treatment being administered (unpublished observation).[48]

Perianal gland adenomas. Perianal gland adenomas are observed in adult male dogs, with adenomas developing about 4.5 times more commonly than carcinomas. Perianal gland adenomas are considered a hormone-dependent disease for which castration (without excision of the gland) can be a successful treatment as it promotes regression without recurrence.[49] We have treated two dogs with perianal gland disease, a 7.1 kg, 16-year old Dachshund Teckel and a 21.5 kg, 12-year old mongrel dog. Both had clinical signs of perianal gland disease characterized by presence of a round, irregular 2-4 cm diameter mass which had developed over the past one to two months. In both cases, administration of an implant of 4.7 mg deslorelin acetate was sufficient to cause a long-lasting regression of the perianal mass without any additional treatment (Romagnoli, unpublished observation).

Treatment of azoospermia due to hypersecretion of FSH

In humans and animals, FSH plays an important role in promoting and maintaining spermatogenesis by binding to specific receptors on the Sertoli cells, thereby stimulating the Sertoli cells to closely interact with germ cells allowing a normal spermatogenic process. Secretion of FSH by the pituitary is regulated by testicular androgen production and, at least in humans and rats, by inhibin which is secreted by the Sertoli cells.[50] In men with primary testicular disorders, Sertoli cell dysfunction is suspected based on an increase in FSH levels and confirmed on histology of a testicular fine needle aspirate or biopsy.[51,52] Although there is little information on the role of FSH on canine azoospermia, FSH plays an important role in spermatogenesis in the canine, and chronically elevated FSH levels are likely to be associated with spermatogenic arrest in the dog like in other species. In men with a) severe oligozoospermia, b) high FSH concentration and c) low Sertoli cell function (evidenced by inhibin B secretion or testicular histology), suppression of the high endogenous FSH levels with the use of a GnRH agonist combined with low exogenous FSH administration causes a rise in inhibin B production reflecting an improvement of Sertoli cell condition with positive effects on spermatogenesis.[53] In the dog, the main source of inhibin is not the Sertoli cell but rather the Leydig cell,[54] therefore it is not clear whether the specific type of azoospermia treated in men by Foresta et al[53] with a combined GnRH agonist-FSH therapy could benefit from the use of a GnRH agonist. However, as we gain more information on canine azoospermia, canine Sertoli cell function

and source of inhibin secretion, a similar clinical application may become useful in the future also in the dog.

Side effects of GnRH agonists

The use of GnRH agonists in the dog is considered safe. No immediate side effects are generally noticed following implant placement, and no adverse effects have been reported in long-term studies performed in male dogs.[7,9,55] As the prolonged use of a GnRH agonist causes a chemical castration, one might think that side effects of both types of castration could be the same. From this point of view, treating a dog with a GnRH agonist implant might be considered as a way of checking on side effects of neutering before actually performing gonadectomy.[38] However, the effects of a chemical castration differ from the effects of a surgical castration in that the latter is characterized by high serum gonadotropin concentrations (because of the lack of negative feedback from gonadal steroids on pituitary release of gonadtropins), while in individuals treated with a GnRH agonist both gonadal steroids as well as pituitary gonadotropins are absent from the general circulation.

Incidence and type of side effects following chronic administration of GnRH agonists have not been studied in dogs. However, the rather common use of leuprorelin and other GnRH agonists as a chronic treatment in men with prostatic cancer has allowed investigation of this problem highlighting a wide range of side effects related to muscle and bone metabolism, glucose homeostasis, endocrine and reproductive function as well as some local skin reactions at the site of implant. Gonandotropin releasing hormone agonists decrease bone mineral density and increase fracture risk, increase weight and fat mass, and decrease lean body mass thereby affecting muscular strength.[56] Insulin resistance has also been reported in men undergoing chronic treatment thus raising the concern that GnRH agonists may also increase the risk of diabetes mellitus and cardiovascular disease.[57] Subclinical pituitary tumors present at the time of treatment may be stimulated to progress: pituitary adenomas in men treated with a GnRH agonist have been reported to grow and cause symptoms of intracranial hypertension.[58] In men with spermatogenic failure undergoing short-term treatment with Leuprorelin (four months) loss of libido and erectile dysfunctions are occasionally observed towards the end of the treatment period.[53] Currently, it is recommended that routine use of GnRH agonists in men with long life expectancy should be carefully evaluated weighing advantages and disadvantage of GnRH agonists (still considered an excellent treatment for prostate cancer) vs other types of treatment such as GnRH antagonists, androgen antagonists or estrogen agonists.[57]

In humans, "*Additional research is needed to characterize better the unintended effects of androgen deprivation therapy and develop optimal strategies to prevent osteoporosis, obesity and obesity related disease*".[59] Although the same consideration applies to our canine patients, musculo-skeletal side effects of GnRH agonists (especially osteoporosis) in dogs are likely to be less relevant than in men because of shorter life expectancy, and occurrence of local skin reaction has not been reported so far in our patients. However, side effects on glucose metabolism should probably be investigated in the dog. Furthermore, although incidence of pituitary tumors in the dog is very rare, pituitary adenomas constitute approximately 80% of the causes of canine Cushing's disease.[60] Almost all of the dogs with Cushing's disease are $\geq$ 6 years of age, and more than 75% of them are $\geq$ 9 years of age.[60] As this is the same age range in which canine BPH is more common, a thorough collection of history and a complete cell count and serum biochemistry are warranted prior to administration of a GnRH agonist implant for the treatment of BPH in adult to older dogs.

Conclusions

Several factors make GnRH agonist compounds unique:

a) the novelty of their pharmacological mechanism, which achieves a downregulation of the HPG axis without "adding" any other hormone, but rather "removing" all hormones from the general circulation;

b) the highly selective inhibitory action at the level of the pituitary gonadotropes

c) the good efficacy in blocking female steroid hormone secretion and the high efficacy in blocking androgen secretion

d) the reversibility of their effects on gonadal secretion which makes their administration a "reversible chemical castration".

In the bitch, the use of GnRH agonists is still characterized by the hardly avoidable side effect of estrus induction. The administration of a progestogen a few days before the placement of an implant appears to work, although it makes the use of such agonists cumbersome and less applicable for contraceptive purposes in the bitch. Furthermore, the prolonged release of GnRH (prior to down regulation) causes prolonged P4 secretion which may worsen a subclinical cystic endometrial hyperplasia causing a clinical uterine condition (which would probably have developed anyway albeit in a longer time). Such sequence of events may be erroneously perceived by the owner as due to the treatment. Therefore, the use of a depot GnRH agonist to inhibit reproductive cyclicity in adult or older bitches should not be decided without making sure that the owner has fully understood advantages and (potential) disadvantages of such treatment.

It should be noted that veterinary GnRH agonist preparations are long-term compounds whose length of action is 6-12 months. This makes these drugs interesting for human doctors as GnRH agonist-based drugs available on the human market only last for a few weeks. The possibility to induce a temporary sterility might be interesting as a way to achieve contraception in men. Therefore, small animal clinicians should be aware that their misuse in humans would carry serious ethical as well as legal implications.

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Inherited diseases of the reproductive tract in dogs and cats

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Introduction

Many veterinarians encounter an abnormality of sexual development during the course of routine practice, particularly during surgery for neutering dogs and cats. Theriogenologists are also likely to encounter these through their evaluations of infertile dogs and cats, and we are motivated to reach a definitive diagnosis. In addition to helping the owner and breeder, this careful "phenotyping" is also needed to further research, with the goal of determining the genetic basis of these disorders. Such research has led to the development of practical genetic tests for other inherited disorders and is proving successful in reducing production of affected cats and dogs.

To simplify the diagnostic plan, we can categorize affected animals according to the first step in sexual development that is identified as abnormal. These were identified as either errors in chromosomal sex, gonadal sex, or phenotypic sex.[1] In that scheme phenotypic sex includes the internal and external genitalia, but excludes the gonad. This is still a practical plan, but requires incorporation of the new nomenclature for disorders of sexual development that was first developed for human patients[2] is now used for animals.[3,4] In addition to incorporating molecular diagnosis, the new nomenclature replaces older terms that are outdated and/or confusing. For example, the general term *intersex* has been replaced with d*isorder of sexual development* (*DSD*), and the terms hermaphrodite and pseudohermaphrodite have been eliminated. Disorders of sexual development are now divided into three main categories: sex chromosome DSD, XX DSD, and XY DSD. The examples below illustrate how the diagnostic plan and the new nomenclature can be integrated to reach a definitive diagnosis. Previous terminology is included for reference purposes. Other canine and feline examples are also reviewed.[3]

Keywords: Disorder of sexual development, inherited disease, abnormal karyotype

Basic steps in the diagnostic plan

1. Obtain a karyotype. If indicated, obtain molecular tests to confirm the presence of genes located on the sex chromosomes.
2. Obtain gonadal histology to confirm the gonadal sex.
3. Identify all internal genital organs present. If indicated, confirm with histology.
4. Identify the external genitalia.
5. To reach a tentative diagnosis, identify the first step that is abnormal. Is it at the level of the sex chromosomes, the gonads, the internal genitalia or the external genitalia?
6. Confirm the diagnosis by DNA testing, or other molecular and/or functional tests.

Sex chromosome DSD

Abnormal karyotype

The normal cat karyotype is 38,XX or 38,XY, while that of dogs is 78,XX or 78,XY. If the karyotype is abnormal, the diagnosis lies in the category of sex chromosome DSD. These are caused by abnormalities in the number or structure of the sex chromosomes. The clinical diagnostic plan should include a description of the internal and external genitalia, and gonadal histology.

Well-known examples of sex chromosome DSD include the XXY syndrome and X monosomy (XO). XXY cats and dogs are sterile, phenotypic males. However, many cats reported have actually been mosaic variants such as XY/XXY and XY/XYY. Most had either a tortoiseshell or calico coat color. Since the orange coat color locus is X-linked in the cat, a coat color containing both orange and black occurs in cats that have at least two X chromosomes, and not in XY males. Therefore these coat colors in a cat with male external genitalia should prompt investigation for XXY or variants. There is no coat color association in dogs, and only four 79,XXY dogs have been reported.

Monosomy X and mosaic variants have been reported in cats and dogs. They present as phenotypic females with primary anestrus. Although short stature may be associated, signs have been variable in mosaic variants.

Trisomy X (79,XXX) has been reported in dogs. Most presented as phenotypic females with primary anestrus. However, two reported had abnormal estrous cycles, including shortened interestrus intervals, persistent estrus, and anovulation, but failed to become pregnant when bred.[5] Both ovaries in each were hypoplastic, suggesting sex chromosome mosaicism in the gonads (78,XX/79,XXX). Only one feline mosaic variant (37,X/39,XXX) has been reported, which was pregnant at presentation.[6]

Normal karyotype

If the karyotype is that of a normal female (38,XX or 78,XX) or a normal male (38,XY or 78,XY), this places the diagnosis in the category or XX DSD or XY DSD, respectively, and not sex chromosome DSD. The next step is to evaluate gonadal histology to determine if there is an error in gonadal sex.

XX DSD

The karyotype is 78,XX, but the gonads are ovotestes or testes. If the karyotype is 78,XX but the gonad contains seminiferous tubules characteristic of the testis, then the first step that is abnormal is in the determination of gonadal sex. The tentative diagnosis is XX DSD, ovotesticular or testicular, which replaces the terms XX sex reversal, XX true hermaphrodite and XX male. After obtaining histologic confirmation of ovotestes or testes, the next step is to test for the presence or absence of *SRY* in the genomic DNA. Finally, the internal and external genital organs, and location of the urethral orifice should be identified. This type of XX DSD has been reported in dogs, but not in cats.

Detailed studies on ovotesticular and testicular XX DSD in dogs from a research pedigree derived from the American cocker spaniel (ACS) confirmed that all affected dogs were 78,XX. Affected siblings had ovotestes or testes, and variable external phenotypes.[7] Subsequent studies confirmed that affected dogs were *SRY* negative, ruling out *SRY* translocation as the cause.[8] The previous name for this disorder was *SRY*-negative XX sex reversal. Breeding experiments conducted in the ACS model indicated the mode of inheritance was compatible with sex-limited autosomal recessive inheritance.[7] To date, ovotesticular or testicular XX DSD has been reported in at least 27 canine breeds and one mixed breed.[3] Although dogs in early reports were not tested for *SRY*, because a test was not available until 1995,[9] all cases tested since have been *SRY*-negative. The causative mutation is likely to be identical by descent in closely related breeds, such as English and American cocker spaniels (genetic homogeneity), but may be different in other breeds (genetic heterogeneity).

The phenotype of affected dogs varies widely. In the ACS model, approximately 10% of affected dogs had bilateral testes, which were often cryptorchid. These dogs were previously called XX males. Epididymides and a complete uterus were also present. In histologic sections, the vas deferens was often identified running parallel to the uterine horn. The penis and prepuce were caudally displaced and exhibited mild hypospadias. The remaining 90% of affected dogs in the ACS model usually had bilateral ovotestes, but occasionally had an ovary and ovotestis, and rarely a testis paired with an ovotestis. These dogs were previously called XX true hermaphrodites. Internal genitalia in these dogs included an epididymis or an oviduct, or both, adjacent to the ovotestes, and a complete uterus. Externally, 15% of dogs with ovotestes had a prepuce-like vulva and 15% had an enlarged clitoris containing a bone. The remaining 70% of dogs with ovotestes had an apparently normal vulva. Subsequently, a narrowed caudal vagina has been identified in dogs with this phenotype. Corrective surgery may be needed to alleviate clinical signs related to hypospadias or clitoral enlargement.

Most affected dogs from the ACS model pedigree have been sterile. However, some have exhibited estrous cycles, and some produced offspring even in the presence of a narrowed vagina or an enlarged clitoris containing a bone. Nevertheless, to prevent production of affected dogs and reduce the carrier frequency in any breed, affected dogs should be removed from the breeding population. Male (78,XY) carriers of the trait and obligate heterozygote carrier females have been fertile. However, further

breeding of the parents of affected dogs should be discouraged to prevent production of affected dogs and reduce the frequency of the mutation within the breed.

The karyotype is 38,XX or 78,XX and the gonads are ovaries, but the internal or external genitalia are fully or partially male. If histology confirms that the gonads are normal ovaries, tentative diagnosis is reached by identifying the first step thereafter that is abnormal. If male genitalia are present, this suggests a tentative diagnosis of XX DSD, androgen excess. As a developing fetus, these animals have been exposed to androgens, which were either produced by the developing fetus itself, or crossed the placenta from the maternal into the fetal circulation.

XX DSD, androgen excess, fetal origin. The most common cause of this type of human XX DSD is an enzyme defect in the adrenal steroidogenic pathway. However, these have not been reported in dogs, and only one case of adrenal enzyme deficiency (11-beta hydroxylase deficiency) has been identified in the cat.[10] The affected domestic shorthaired cat had a calico haircoat and male external genitalia, but testes were not palpable within the scrotum at six months of age. The karyotype was that of a normal female (38,XX). A complete uterus and bilateral ovaries were removed at laparotomy. In addition to the ovaries, oviducts and uterus, histology confirmed the presence of epididymides and deferent ducts. As development of the latter two organs is dependent upon testosterone stimulation, and development of male external genitalia is dependent upon dihydrotestosterone, which is produced from testosterone, together these findings are indicative of XX DSD, androgen excess. The androgen source was next investigated.

At ten months of age, penile spines were present, which in the cat, are dependent upon sustained androgen stimulation. Polydipsia, polyuria, and male urinary marking behavior were also present. These signs indicated that the cat was still producing testosterone, and resting serum testosterone concentrations were within the normal range for a male cat. High resting serum ACTH concentrations and low serum cortisol concentrations after ACTH stimulation suggested an adrenal enzyme deficiency. Elevated serum progesterone, 17-hydroxyprogesterone, androstenedione, testosterone, deoxycorticosterone and 11-deoxycorticosterone concentrations indicated that a defect in 11-beta hydroxylase activity was likely. Subsequent to maintenance prednisone therapy, serum testosterone concentrations decreased and clinical signs ceased.

When 11-beta hydroxylase is deficient, cortisol levels are insufficient to exert negative feedback on ACTH production. This leads to adrenal hyperplasia and excessive production of steroid precursors, which are shunted to alternate enzymes in the pathway, terminating in testosterone production. Prednisone therapy in this cat was apparently sufficient to suppress ACTH secretion. A number of steroid enzyme deficiencies have been reported to cause human congenital adrenal hyperplasia. Such individuals can present with varying degrees of glucocorticoid and mineralocorticoid deficiency as well as androgen excess.

XX DSD, androgen excess, maternal origin. These disorders have not been reported in the cat. However there are several research and clinical reports in dogs where females were masculinized by steroids administered to their pregnant dam. These preparations included mibolerone, testosterone, and progestagens.[11-14] As adult responses to steroids are not reliably predictive of fetal response, it is inadvisable to administer steroid preparations to pregnant dogs or cats, and particularly during the period of internal and external genital development.

The karyotype is 38,XX or 78,XX, the gonads are ovaries and the genitalia are unambiguously female, but abnormal. The first step that is abnormal is in the female genitalia. These abnormalities are included under XX DSD, other: Mullerian agenesis/hypoplasia. The phenotype is clearly female, as there are no male genitalia present, but the vagina or uterus has failed to develop properly. In humans, such defects are highly associated with renal agenesis and/or ectopy, and cervicothoracic somite dysplasia. This syndrome is termed MURCS for Mullerian Duct Aplasia, Unilateral Renal Agenesis and Cervicothoracic Somite Anomalies (Online Mendelian Inheritance in Man, number %601076). If one component of MURCS is identified in a human patient, this prompts evaluation for the other anomalies.

A syndrome similar to MURCS may occur in cats and dogs. In a survey of 53,258 cats and 32,660 dogs undergoing elective ovariohysterectomy, congenital uterine abnormalities were identified in 0.09% of female cats and 0.05% of female dogs.[15] These included unicornuate uterus, segmental aplasia of one uterine horn and uterine horn hypoplasia. In 29.4% of cats and 50% of dogs with uterine abnormalities in which the kidneys were also evaluated, ipsilateral renal agenesis was present. Although cervicothoracic abnormalities were not described in this report, careful evaluation of such cases could establish the prevalence of Mullerian agenesis and hypoplasia as isolated defects, the prevalence of their association with ipsilateral renal agenesis, and whether the full MURCS syndrome occurs in cats and dogs.

XY DSD

The karyotype is 38,XY or 78,XY but at least one gonad is an ovotestis. In this case, the first step that is abnormal is in the determination of gonadal sex. The tentative diagnosis is XY DSD, ovotesticular, which replaces the terms XY sex reversal and XY true hermaphrodite. The next diagnostic step is to confirm the presence or absence of *SRY* in the genomic DNA. Finally, the internal and external genital organs, and location of the urethral orifice should be identified.

One case of feline XY DSD, ovotesticular has been confirmed.[16] The year old mixed breed cat presented as a phenotypic male with bilateral cryptorchidism. The *SRY* gene sequence was the same as in a normal male control. Bilateral ovotestes were located at the caudal pole of the kidneys. Epididymides were adjacent to each gonad and partially developed vasa deferentia, along with a complete bicornuate uterus and oviducts.

The karyotype is 38,XY or 78,XY, the gonads are testes, but female genitalia are present in regions dependent upon androgens for masculinization. The first step that is abnormal is androgen dependent masculinization, indicating a tentative diagnosis of XY DSD, disorder in androgen synthesis or action. The most commonly reported disorders of this type are androgen receptor defects.

1) When the androgen receptor is nonfunctional, there is complete failure of masculinization in the internal and external genitalia (complete androgen insensitivity syndrome, or CAIS).

One case of feline CAIS has been reported.[17] The external genitalia were unambiguously female at six months of age when the cat was presented for ovariohysterectomy. Testes were located just caudal to the kidneys, and there were no Mullerian or Wolffian duct derivatives present. The testes contained seminiferous tubules and abundant Leydig cells. Inability of the androgen receptor to bind androgens was demonstrated *in vitro*.

2) When the androgen receptor is functional to some degree, there is variable masculinization of the internal or external genitalia (partial androgen insensitivity syndrome, or PAIS).

One case of canine PAIS has been reported in a mixed breed that was phenotypically female at six months of age.[18] Later, scrotal-like swellings containing testes were identified on each side of the vulva. Spermatogenesis was absent in both hypoplastic testes. A well-developed epididymis and partially developed deferent duct were adjacent to each testis. Abnormal androgen binding to the androgen receptor was demonstrated *in vitro*.

The karyotype is 38,XY or 78,XY, the gonads are testes, the external genitalia are male, but a uterus is present. The first step that is abnormal here is in Mullerian duct regression. Although classified under the category of XY DSD, other, these are disorders in the synthesis or action of Mullerian inhibiting substance (MIS), also known as anti-Mullerian hormone (AMh). The example below has the specific diagnosis of persistent Mullerian duct syndrome (PMDS). Mutations in *MIS* or its Type II receptor (*MISRII/AmhR2*) cause the same PMDS phenotype in human patients.

Persistent Mullerian duct syndrome in the miniature schnauzer has been reported frequently and in several continents.[3] Externally, PMDS dogs are unambiguously male, except that approximately 50% are unilaterally or bilaterally cryptorchid. In addition to having complete male internal and external genitalia, these dogs also develop all Mullerian duct derivatives. Bilateral oviducts and epididymides are adjacent to the testes. The deferent ducts are included in the lateral walls of the uterus, and the cranial end of each uterine horn is attached to the caudal pole of the ipsilateral testis. The cervix is present, and

the cranial vagina terminates within the craniodorsal aspect of the prostate gland. Radiographic contrast studies in some PMDS dogs confirmed a patent connection between the cranial vagina and the prostatic urethra. Pyometra and neoplasia in cryptorchid testes are common sequelae.

Cryptorchidism and infertility are not consistently associated with PMDS in the miniature schnauzer. Fifty percent of PMDS dogs had bilateral scrotal testes and externally appeared to be normal males. Such dogs could easily escape clinical recognition. The remaining 50% of PMDS dogs were cryptorchid. The close attachment of the testis to the uterine horn likely interferes with testis descent. PMDS dogs with at least one scrotal testis were fertile.

Persistent Mullerian duct syndrome in the miniature schnauzer is inherited as a sex-limited, simple autosomal recessive trait.[19] The causative mutation in the MIS type II receptor has been identified[20] and a DNA test is available to detect affected, carrier and noncarrier miniature schnauzers.[21] Persistent Mullerian duct syndrome has also been reported in the basset hound in Europe and a mixed breed dog, but in those cases, the causative mutations are unknown.

The karyotype is 38,XY or 78,XY, the gonads are testes, the external genitalia are male, but there is abnormal development of the male urethra. The first step that is abnormal occurs in only one portion of the external genitalia. Although classified under the category of XY DSD, other, the specific diagnosis is isolated hypospadias. This disorder has been rarely reported in cats and dogs, and no molecular etiology has yet been identified in either. Two reports in Himalayan cats described the severe phenotype.[21,22] The scrotum is bifurcated by a urethral canal that is open along the entire dorsal aspect, and the penis and prepuce are diverted dorsally. Canine isolated hypospadias of varying severity has been reported, where the urethral orifice can be located in the glans penis, the penile shaft, or the perineum. The Boston terrier had the highest prevalence of isolated hypospadias in a survey of veterinary hospitals.[23]

The karyotype is 38,XY or 78,XY, the gonads are testes, the external genitalia are male, but one or both testes have failed to descend into the scrotum. The first step that is abnormal occurs in only one portion of the external genitalia. Although classified under the category of XY DSD, other, the specific diagnosis is isolated cryptorchidism, which is different from cryptorchidism that is associated with other DSD. In breeds where cryptorchidism has been associated with other DSD, such as PMDS in the miniature schnauzer (above), affected dogs can be screened for those mutations to obtain a definitive diagnosis.

The following discussion is limited to isolated cryptorchidism, wherein XY males are phenotypically male in all respects except that one or both testes are undescended. The undescended testis may be located anywhere from the caudal pole of the kidney to the inguinal canal, or external to the canal but cranial to the scrotum. Thus, cryptorchidism is a term encompassing several phenotypic categories, likely reflecting a genetically complex control of testis descent.

In cats, scrotal testes are not easily palpable in young kittens, so cryptorchidism is often identified in males presented for neutering before one year of age. Prevalence has been described as 1.7%[24] and 1.3% of cats presented for neutering,[25] with most being unilaterally cryptorchid. Prevalence in Persian cats was reported to be significantly greater than in other breeds.[24]

Canine testes are undescended at birth. At the end of gestation, the testis lies on the peritoneal side of the internal inguinal ring, but passes through the inguinal canal within ten days after birth.[26] However, it is unclear when the canine testis becomes secured to the scrotum. Clinical diagnosis is warranted if testes are undescended by six to eight weeks of age. In contrast to the cat, canine cryptorchidism is prevalent, ranging from 6.8% of males presented for neutering[25] to 1.4% of dogs at six to twelve months of age.[27] It is also more prevalent in some breeds.[25,27,28] In one study, inguinal cryptorchid testes were most common.[25]

As in humans, late testis descent has been identified in dogs. In one study of cryptorchid dogs examined regularly until one year of age, late descent occurred in 24.6% of cryptorchid testes, with 63.3% of those being unilaterally cryptorchid.[1] However, of the 24.6% that descended, most did so by 14 weeks of age and none descended after six months of age. An increased risk of neoplasia in undescended testes

is well documented, estimated as 12.7/1000 dog-years at risk.[27,28] Consequently castration of affected dogs is recommended, which also serves to reduce the frequency of cryptorchidism in the population.

The molecular basis is unknown for the varying phenotypes constituting canine cryptorchidism, but is likely to be polygenic. In addition, cryptorchidism may be genetically heterogeneous between breeds. The genetic etiology is being pursued with association studies[29] and candidate gene studies.[30]

Conclusions

By following the basic steps in the diagnostic plan and identifying the first abnormal step in sexual development in a clinical case, we can narrow the tentative diagnosis to an error in chromosomal sex, gonadal sex or phenotypic sex. This allows us categorize the abnormality as a sex chromosome DSD, XX DSD or XY DSD, which narrows the list of diagnostic tests needed to reach a definitive diagnosis. Definitive diagnoses are important to the owners and breeders, who can benefit from genetic counseling for their breeds. A definitive diagnosis also provides the accurate phenotype information that is needed for further research to identify causative mutations, create DNA tests, and ultimately reduce production of affected animals. Incorporation of the new DSD nomenclature into our communications should lead to increased collaboration between veterinarians, researchers, and physicians, to the benefit of animals and humans.

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Canine autoimmune orchitis

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Abstract

Autoimmune orchitis is defined as autoimmune inflammation of the testis with or without the presence of anti-sperm antibodies and will result in infertility in both males and females. It is a rarely described disorder in dogs that may lead to permanent infertility. However, non-invasive diagnostic tools has been lacking and thus, this condition may be under-diagnosed. Clinical signs include infertility and sperm abnormalities. Diagnostics include a thorough workup for infertility including history, general and reproductive tract-specific physical examination, blood work, urinalysis, and ultrasound. Antisperm antibodies may be present, but their significance is still disputed. Testicular biopsies reveal lymphocytic infiltrates progressing from the straight tubules to the rete testes and the efferent ducts eventually resulting in necrosis and the absence of spermatogenesis. If no underlying causes for the autoimmune orchitis are discovered and testicular atrophy has not occurred, treatment with immune suppressive agents have been suggested. If anti-sperm antibodies are present and the patient still produces some sperm, direct surgical intrauterine insemination should be considered to increase fertility rates.

Key words: Autoimmune orchitis, orchitis, canine, testis, infertility, antisperm antibodies

Introduction

Autoimmune orchitis is defined as autoimmune inflammation of the testis with or without the presence of anti-sperm antibodies and will result in infertility in both males. Autoimmune orchitis can be classified as either primary or secondary depending on its cause. In primary autoimmune orchitis, antisperm antibodies are antibodies against testicular tissues, which are present in the absence of systemic disease. Secondary autoimmune orchitis is generally associated with systemic autoimmune disease.[1] Dogs and mink are the only species in which spontaneous autoimmune orchitis has been shown to occur.[2-4] It can be induced in guinea pigs and rabbits by vasectomy. A disease similar to autoimmune orchitis has been described in mice after thymectomy shortly after birth. It can also be induced in mice and other species by injection of homologous sperm or testis antigen.[4-6] Histologic changes similar to those found in autoimmune orchitis have been described in humans and have been associated with infertility in some cases. In humans, most cases of autoimmune orchitis are due to either trauma in the broadest sense to testes or vas deferens (torsion, obstruction, vasectomy) or systemic immune mediated disease (lupus, chronic rheumatic disease).[1]

Early studies of autoimmune orchitis in dogs were performed in a beagle colony with lymphocytic thyroiditis and concurrent orchitis. Dogs were observed to become infertile during the first two years of stud service. Initially, the dogs were shown to be normospermic, then oligospermic, and finally, aspermic.[3] This study suggests a genetic predisposition, as the more severe the changes were, the more closely these dogs were related. Dogs in the same study without lymphocytic thyroiditis or orchitis were not related to the affected dogs. Further evidence of a genetic component was demonstrated in a study of two related dogs (sire and son) that had the same testicular pathology and autoantibodies. Interestingly, these serum autoantibodies did not bind to the testes of other dogs. Deposition of IgG and C3 was demonstrated around interstitial capillaries in the testes and occasionally on sperm tails in the related dogs.[2]

The incidence of autoimmune orchitis in dogs is not known, perhaps because testicular biopsies are not often performed and the detection of antisperm antibodies is not commonly done. In humans, immune responses within the testes have not been well studied either. However, the incidence of serum and/or seminal fluid antisperm antibodies in primary autoimmune orchitis has been estimated to be between 7 and 12% of infertile men and women.[1]

Clinical signs

In dogs with spontaneous or induced autoimmune orchitis, decreased fertility or infertility was the most common complaint. In a few affected beagles, the testes were palpably slightly smaller than in unaffected dogs.[3] Semen evaluation in affected dogs may reveal oligospermia, teratozoospermia, azoospermia, aspermia or sperm agglutination.

Diagnostics

Even if autoimmune orchitis is suspected, a complete workup for infertility should be performed. A complete history is imperative and should begin with general information such as vaccination history, previous illnesses, trauma, current and previous medications, supplements, and environmental information such as number of dogs and other animals in the household and use of the dog (field trial or hunting dog, show dog, etc.). Information specific to reproductive health should also be obtained, such as previous or current episodes of potential hyperthermia to the testes or scrotal swelling or dermatitis. Breeding history also provides information as to the degree of infertility. The age at which the first attempts at breeding were undertaken should be assessed as well as the number of matings performed, the number of litters sired, timing of the bitch used, and if the stud in question was bred to a proven female. Results of recent brucellosis testing should also be reviewed.

The physical examination should include a general examination followed by the male reproductive examination. Libido and semen evaluations are performed before uncomfortable things such as prostatic examination are done. Briefly, the testes should be examined for size, shape, and consistency. The head, body, and tail of the epididymides should be carefully palpated and the prostate is examined for symmetry, size, and pain. Testes, epididymides, and prostate can all be evaluated by ultrasonography. Laboratory tests include a complete blood cell count, a serum biochemistry screen, urinalysis, antisperm antibodies,[7-10] and complete semen evaluation. Alkaline phosphatase may be measured in the ejaculate to assess if the collection is complete. While bacteria to some degree are always present in the ejaculate regardless of fertility status, culture and sensitivity may be considered if there are clinical signs of infection or if neutrophilia and toxic neutrophils are present in the ejaculate.[11]

If autoimmune orchitis is suspected, testicular cytology or biopsy may be considered.[12-14] The advantage of cytology is that the procedure is quick, simple, and leaves minimal damage. On the other hand, the disadvantage is a low yield of cells and progression of spermatogenesis cannot be evaluated. In general, Leydig cells cannot be assessed nor can the architecture of the testicular tissue. Side effects of a needle biopsy may include minor hemorrhage, minimal scrotal swelling and erythema, cellular degeneration and necrosis, and in one case testicular atrophy was noted. In most cases, the lesions remained focal and did not have any effect on sperm quality, testicular size, or testosterone concentrations.[12,13] Testicular biopsies can be performed either by surgically removing a wedge or by using a triggered biopsy instrument, such as a needle punch biopsy device. The advantages are detailed assessment of the testicular tissues including progression of spermatogenesis and architecture of the tissue. Side effects include hemorrhage, maturation arrest, and focal fibrosis and the development of sperm granulomas. The presence of antisperm antibodies have been documented after testicular biopsy. However, these antibodies disappeared rapidly after biopsy and no significant long-term changes in sperm quality and motility were detected.[7] In humans, immune responses after biopsy have been shown to be minimal and of no clinical significance.[1]

Pathophysiology

Experiments were performed to characterize antibody responses to sperm in dogs that were chronically infected with *B. canis*. The results revealed sperm agglutinating antibodies both in serum and in seminal fluid that were highest in those dogs infected for four to six months. The chronically infected dogs were skin tested with sperm extracts and showed delayed type hypersensitivity reactions. The most severe reactions were seen in those dogs with testicular atrophy. Semen evaluation demonstrated teratozoospermia and sperm adhering to clusters of inflammatory cells. In this model, it is thought that

the pathogens engulfed by macrophages are able to breach the sperm blood barrier thus presenting sperm cells to the immune system resulting in autoantibody production.[8]

Studies in guinea pigs revealed that injecting sperm homogenates along with Freund's complete adjuvant induces antisperm antibodies and results in the development of orchitis. Similar studies in BALB mice and in dogs using Freund's incomplete adjuvant did not result in a similar outcome. It was subsequently shown that when BALB mice were immunized with sperm homogenates and injected with *Bordetella pertussis* subcutaneously, orchitis could be induced. Based on these studies, dogs received a single intratesticular injection of Bacillus Calmette-Guerin, which resulted in temporary azoospermia within three to six weeks that lasted from six weeks to almost one year. Fertility was assessed by breeding the dogs during the phase of azoospermia and after recovery. No puppies were produced during the azoospermic period but after recovery all dogs sired healthy litters. Interestingly, no antisperm antibodies were detected during any of the azoospermic phases or after recovery. In humans, autoantibodies are often induced upon restoration of vasectomy, but these antibodies rarely result in reduction of fertility. In general, many of the studies show that recovery is likely if there is incomplete degeneration of the germinal epithelium, indicating that aspermatogenic orchitis may not lead to irreversible infertility.[9]

Experimental studies in mice have shown that cell mediated immunity in autoimmune orchitis is conferred through CD 4^+ cells. Antibody mediated responses were evidenced by the presence of antibodies on testicular germ cells outside of the blood testis barrier, which was often present well before cellular infiltration of the testes. Other studies in mice and rabbits have shown immune complexes of testicular antigens and autoantibodies within the testes. In these studies, the progression of orchitis was followed histologically. Initially, focal lymphocytic infiltrates were seen around the straight tubules. Next mild inflammatory infiltrates were noted within the rete testis and/or the efferent ducts. Occasionally, mild dilation of the seminiferous tubules was observed. Eventually, the changes became moderate to severe over time. Final changes included severe inflammation of the testis with necrosis, dilation of the seminiferous tubules and a complete lack of spermatogenesis. These changes were not just localized to the testes but also to the epididymides and the vas deferens, which exhibited a similar progressive pattern of change. In this study, administering antibodies against TNF alpha reduced pathology in the testes to more focal type lesions demonstrating the importance of TNF alpha in the development of orchitis. Epididymitis was also completely inhibited by the administration of antibodies against TNF alpha. In addition, experimental studies of autoimmune orchitis in mice have shown that TNF alpha is produced both in testicular lymphocytes and macrophages leading to progression of disease.[4]

Further studies have shown that TNF alpha, its type I receptor, IL-1 alpha, IL-1 beta, and its receptor are normally expressed in the male gonads. These cytokines have been shown to play an important role in maintaining testicular health. Increasing concentrations of TNF alpha are present in pro-inflammatory responses, immune regulatory responses, and finally apoptosis. In experimental models of autoimmune orchitis, the increased concentrations of TNF alpha result in up regulation of its type I receptor. Sertoli cells and testicular macrophages produce IL-1 alpha. Increased concentrations of IL-1 alpha result in inflammation and induction of other cytokines and enzymes. Cytokines and enzymes induced include TNF alpha and nitric oxide synthase, which themselves have pro-inflammatory properties. Studies indicated that this increased expression of TNF alpha may lead to increased permeability of the endothelium allowing for extravasation of monocytes into the testicular tissues and activation of T cells and macrophages. The macrophages then in turn produce more TNF alpha leading to progression of orchitis.[15]

Treatment

Currently, there is no treatment for autoimmune orchitis. If secondary autoimmune orchitis is suspected, then immune suppressants such as glucocorticoids could be considered.[1] One would have to carefully consider the doses and the duration of treatment and prudently weigh the risks versus benefits. To increase the chances of fertility, at least some sperm cells must be present in the ejaculate. If

antisperm antibodies are present in serum and/or seminal fluid, washing the sperm before insemination may be beneficial. However, once the sperm are bound to the antibodies, they form irreversible complexes and the antibodies cannot be washed off. Another option is to perform intrauterine insemination close to the ovaries or more sophisticated methods such as intracytoplasmic sperm injection. In reality though, by the time dogs are presented for infertility due to autoimmune orchitis, the disease has usually progressed to aspermia and is at that point likely irreversible.

Conclusion

Autoimmune orchitis in dogs is a poorly understood disease. Future studies are needed to understand the pathophysiology and potentially inciting causes. As in other species, the role of antisperm antibodies in the development of autoimmune orchitis remains unclear. This is perhaps due to the fact that there are no reliable, standardized methods of assessing antisperm autoantibodies. Currently, diagnosis of autoimmune orchitis is best made by obtaining a testicular biopsy along with a detailed history, physical examination, and semen evaluation. Future studies will focus on less invasive diagnostics and elucidation of the pathophysiology, so that better targeted therapies may be developed.

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Pedigree analysis and inherited canine & feline reproductive diseases

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Abstract

As theriogenologists, breeders come to us not only for diagnosis and treatment but also with questions pertaining to the genetic nature of a specific disease. The recent advances in molecular genetics and the availability of genetic maps have made it possible to discover the basis of many genetic diseases in the dog and cat. Over 600 inherited diseases have been described in dogs and about 200 in cats, with more genetic diseases being added each year. This has led to the development and growing need for incorporation of clinical genetics into veterinary practice with the reproductive specialist playing an important role in the detection of potentially new genetic diseases and to provide genetic counselling. To provide assistance to the breeder, we as theriogenologists already appreciate the importance of accurately diagnosing a disease and now need to be able to decipher pedigrees along with historical information that will allow us to suggest a reasonable mode of inheritance for a given disease.

Keywords: Pedigree analysis, modes of inheritance, inherited reproductive diseases.

Introduction

The first step to understanding the mode of inheritance is precise phenotyping, i.e. the disease of interest must be accurately diagnosed. For example, two related bullmastiffs are both one year of age; are polyuric and polydipsic; have elevated serum renal values; have a urine specific gravity of 1.011; and a urinary protein to creatinine ratio of 5. However, one has renal dysplasia and the other an inherited glomerulonephropathy.[1] Had the two been lumped together as one disease, the pedigree analysis would have been incorrect. The second step to understanding the mode of inheritance is analyzing the provided pedigrees. Figure 1 shows a typical pedigree of a dog registered with one of the European kennel clubs. Tracking genetic diseases through these types of pedigrees is not easy and it becomes clear that more than just a three-generation pedigree is needed. To visualize inheritance patterns, we typically convert written pedigrees into diagrammatic pedigrees as demonstrated below. Once converted, the inheritance patterns will present as one of the modes if inheritance as outlined. This information is essential in providing a thorough work up of any genetic disease.

Definitions and symbols

A *gene* is defined as the basic element of heredity that determines traits. A gene is transmitted from parents to progeny. An *allele* is an alternative version of a given gene. The chromosomal location at which a gene with a specific function resides is called a *locus*. If pairs of alleles of a given gene are the same, they are called *homozygous* (or *homozygote* when referring to the genotype in the individual). *Heterozygote* means that the pairs of alleles of a given gene are different.

The *genotype* is defined as the genetic constitution and consists of internally coded, heritable information. The genotype could essentially be viewed as the individual's architectural blue print. Usually, only alleles of genes of interest listed, e.g. B for black, b for brown, e for yellow if Labrador hair color is of interest. The *phenotype* is the summary of observable properties of an organism, and the outward, physical manifestation of the genotype. In other words, the phenotype is the implementation of the blue print.

Any gene that is located on a non sex chromosome is considered *autosomal*. Genes on the X or Y chromosome are called *X-linked* and *Y-linked*, respectively. In *dominant* traits, the allele is expressed in the phenotype when present on only one chromosome (in heterozygotes). For *incomplete dominant* traits, the allele is expressed in heterozygotes, but homozygotes are more severely affected and in *complete*

dominance, homozygotes are indistinguishable from heterozygotes. In contrast, in *recessive* disorders, the allele can only be expressed when present on both chromosomes (in homozygotes).

Pedigrees are of great importance in recording the occurrence of a known or suspected genetic disease in families and in determining the mode of inheritance. The recommended method of drawing lines indicative of relationships between relatives in domestic animals differs somewhat from that used in humans. This is because in domestic animals, inbreeding is common and the generations are often overlapping, making the human pedigree format awkward. The basic principle used in drawing animal pedigrees is that a mating is indicated by vertical lines that come out of the bottom of the symbols of the two parents and are connected by a horizontal line on which the offspring are arrayed (Figure 2). To convert the written pedigree, animal names are coded by numbers, their sexes indicated by symbols (squares are males and circles are females), and their relationships indicated by lines (Figure 3). There are commercially available products that assist with drawing pedigrees (e.g. Pedigree/Draw, Southwest Foundation for Biomedical Research, San Antonio, TX) and that calculate coefficients of inbreeding if needed. However, in most cases drawing a quick five to 10 generation pedigree will allow for an educated guess as to the mode of inheritance of a given trait or disease.

Modes of inheritance with examples

Autosomal dominant inheritance

One allele of a given gene is enough to determine the phenotype (trait/disease allele D with d being normal) and since the gene is located on an autosome (non sex determining chromosome) the risk to males and females is equal. Affected individuals are usually heterozygotes (Dd). At least one parent is affected, unless the condition is the result of a new mutation.

Affected x normal matings produce 50% affected offspring. 50% of the animals are normal (phenotype) and are homozygous in the normal allele (dd) and 50 % of the animals are phenotypically affected and are heterozygous for a normal and a trait determining allele (Dd; Figures 4a1 and 4b).

Affected x affected matings (rare) produce 75% affected offspring. Only 25% of the offspring are normal (dd), but 75% are phenotypically affected but genotypically different; 50% are heterozygote (Dd) and 25% are homozygotes (DD; Figures 4a2 and 4b). Double doses of dominant traits often lead to a more severe phenotype that can lead to early morbidity and mortality.

In autosomal dominant disorders that are severely deleterious (have a fitness close to zero), or which would be selected against by breeders, most of the cases observed in a population will represent new mutations. These will occur as rare sporadic cases with no prior evidence of their occurrence in related animals. A number of such mutations affecting collagen metabolism in dogs and cats have been found in recent years.[2] In these, fragility of the skin and joint laxity preclude survival under ordinary circumstances and proof of their dominant nature was obtained only by breeding studies in a protected laboratory environment.

The mating of two individuals with a dominant disorder will be rare, but in such cases the number of offspring may be reduced because the homozygous state is lethal in utero. Owing to the loss of homozygotes (one out of four embryos in matings between two heterozygotes), the proportion of affected offspring at birth will be 2/3 rather than 3/4. Examples include desired traits such as Chinese crested dogs, Mexican hairless dogs, and Scottish fold cats in which the trait is encoded by a single "defective" gene. When the animals with the desired traits are bred together, one quarter of the offspring will be homozygous for that trait, which is then lethal in the hairless dogs and the homozygous Scottish fold cats will have severe cartilage defects.

It should be fairly straightforward to eliminate traits inherited as simple autosomal dominant, if they are easily recognized and their onset occurs before the age of reproduction. In such cases, only the affected the animal has the mutation and it can be removed from the breeding pool before it reproduces. However, the autosomal dominant mode of inheritance is the more rare occurrence. It appears that most disorders, in dogs and cats at least, are inherited as recessive traits that are transmitted by either a single gene (autosomal recessive) or, more likely, multiple genes (complex inheritance). It is more difficult to reduce transmission of disorders that are inherited in this manner.

Autosomal recessive inheritance

An animal has to have two trait determining (disease) alleles to express the phenotype or be affected (rr). Again both female as well as male animals are equally affected. An animal that has one disease allele is phenotypically normal but is called a carrier (for the disease allele, r; carrier = Rr). The normal individual has the "RR" genotype.

Carrier x normal mating produces 50% carrier offspring. 100% of the animals are of a normal phenotype. However, 50% of these are homozygous in the normal allele (RR) and 50 % of the animals are carriers (Rr) and thus heterozygous for a normal and a trait determining allele Figures 5a1 and 5b).

Carrier x Carrier matings produce 75% phenotypically normal offspring. However, 2/3 of these are carriers (Rr, rR, RR). Without specific tests it is often impossible to distinguish the normals from the carriers; 25% of the offspring are affected (rr; Figures 5a2 and 5b).

Autosomal recessive inherited diseases are by far the most common class of single gene disorders in domestic animals. In affected families, most affected animals are born to clinically normal parents that are carriers of a mutant allele that has been inherited from an ancestor that is common to the sire and the dam (some degree of inbreeding is present). Often when a "new" disease shows up in a dog or cat, the breeders tend to discount autosomal recessive inheritance because this "new" disease has not been seen in over five generations. However, as evident in Figure 5c, the original mutation may have occurred many generations ago but it wasn't until the sixth generation that two carriers came together to produce an affected animal.

As theriogenologists, we are most concerned with disorders that affect the reproductive tract, which brings additional problems to the analysis, such as sex-limited expression of a particular disorder. That is, the disorder causes an abnormal phenotype in one sex, not both, so sterility or infertility only occurs in one sex. Another example of sex-limited expression is cystinuria in Newfoundland dogs, which is present in both males and females but it is almost always the affected male dog that presents to the clinic because the cysteine stones lodge in the narrow male urethra and cause blockage.[3,4]

The other, unaffected, sex can continue to transmit the mutation. Since the genotype is not expressed in the phenotype of one, it makes pedigree analysis difficult even if the disorder is inherited as a simple Mendelian autosomal recessive trait: the parent that cannot express the phenotype, although it looks normal, can be carrying one or two copies of the mutation, or it could have two normal copies of the gene. To deduce the genotype of the parent that cannot express the trait, one would need to perform experimental matings with progeny testing. This is a good reason to support research to identify the causative mutation, create a DNA test, and genotype animals directly. Another problem is that a disorder can have a severe phenotype in some cases, and a less severe phenotype in others. A disorder that exemplifies both types of pedigree problems is persistent Müllerian duct syndrome in the miniature schnauzer.[5] Females are unaffected by this mutation, as are male carriers. Males that are homozygous for the mutation have normal testes and internally have oviducts, a complete uterus, a cervix and cranial vagina. Only half the affected dogs have associated cryptorchidism, which often elicits clinical investigation, while the other half have scrotal testes, and thus appear to be normal males.

X-chromosomal recessive inheritance

The gene of interest is located on the X-chromosome: therefore two copies of the trait determining (disease) allele are necessary in females but only one copy is needed in males for the phenotype to be expressed.

Affected males are hemizygotes (they only have one X chromosome). Most affected offspring are males, born of matings between carrier females and normal males. In such matings, 50% of the sons are affected and 50% of the daughters are carriers. Affected females only occur as the result of matings between affected males and carrier or affected females. When the male is the only affected parent, male to male transmission of the condition is never observed.

Carrier female x normal male matings result in 50% of the males being affected (X^cY); 100% of the females and 50% of the males are of a normal phenotype but half of the females are carriers (XX^c)(Figures 6a1 and 6b).

Carrier female x affected male matings (rare) produce 50% affected offspring. Half of all males are affected (X^cY) and the other half normal (XY); whereas half of all females are carriers (XX^c) and the other half affected (X^cX^c)(Figures 6a2 and 6b).

In domestic animals, an important feature of X-linked recessive disorders is that in matings of carrier females to normal males, one half of the male offspring will be affected, regardless of whether the male (sire) is related to the female. Thus, inbreeding is not a prominent feature in X-linked recessive disorders. This is in contrast to autosomal recessive inheritance, in which inbreeding is often present, the parents of affected offspring having inherited the mutant gene from ancestors which they share. Examples of X-linked recessive diseases include severe combined immunodeficiency in the Corgi and Basset hound[6,7] and ectodermal dysplasia in various breeds (usually a new mutation).[8]

X-linked dominant inheritance

X-linked dominant traits are more commonly found in females than males (twice as common in rare traits, since females have two chances to receive an X with the mutant allele, while males have only one). If the mutant allele is lethal to hemizygous male embryos, the disorder will be found only in females. The chief characteristic of X-linked dominant inheritance in families is that affected males transmit the trait to all of their daughters and none of their sons. Affected females are usually heterozygous and it may not be possible to distinguish between autosomal dominant and X-linked dominant inheritance from their offspring. One half of the females and one half of the males will be affected in both cases. However, this would be the case only when dominance is complete. That is, when the effect in a heterozygous female is essentially the same in a hemizygous male. An example of X-linked inheritance in which the mutation is "incompletely dominant" in heterozygous females is found in a form of hereditary renal disease in Samoyed dogs. In this case, females with the mutation on one X chromosome have a slowly progressive form of glomerular defect and usually survive for long periods. Males, whose only X contains the mutant gene, have a much more severe disorder, rapidly progressing to renal failure and death by 15 months of age. This disorder has been shown to be due to a mutation in the X-chromosomal gene that encodes the alpha-5 chain of type IV collagen, a constituent of the glomerular basement membrane.[9]

Complex mechanisms and complex modes of inheritance

Many diseases that are of great concern to both breeders and veterinarians are caused not by a single gene but by the interactions of several genes (Figure 7). To make matters more difficult for the breeder and the geneticist, the phenotype (or the appearance of the trait or disease) can often be modified by environmental influences such as nutrition or exercise. In other words, complex or polygenic disorders are caused by mutations or sequence variations in just a few or many genes with the interaction of the environment and are thus more difficult to evaluate. Complex traits may occasionally have a monogenic basis, in that a DNA repair gene suffers a mutation causing mutations in other genes. Other examples of monogenic diseases mimicking complex traits include diseases with variable expression or incomplete penetrance. The variable expression of these diseases with single gene disease-causing genotype may be due to environmental influences as well as modification through other genes. In oligogenic traits, only a few different genes are involved in expression of disease. Finally, the most complex of all, some diseases are considered multifactorial, in that a particular disease is caused by many genes, each of small effect but when added convey susceptibility. When exposed to a specific environmental situation, the disease then becomes evident.

In cases of complex inheritance, it is not uncommon for the phenotype to vary in severity between affected animals. Cryptorchidism may exemplify this type of complexly inherited disorder, as the phenotypes include late testis descent, unilateral or bilateral cryptorchidism, and several locations for the undescended testis. In addition, cryptorchidism could be an example of another problem: genetic

heterogeneity. This means that the same phenotype can be caused by mutations in different genes. In that example, we could find that unilateral cryptorchidism is caused by mutations in genes A and B in the Irish setter, while mutations in genes C and D cause the same phenotype in the miniature poodle. In that case, we would need to study both breeds to identify their specific mutations, and design DNA tests specific for each breed.[10]

Conclusion

As theriogenologists, we can make an impact by seeking definitive diagnoses, but also developing diagnostic criteria that distinguish the degrees of severity in the affected phenotype. As expert phenotypers, we can also participate in research studies that require precisely diagnosed cases and controls to identify causative mutations. For example, in Genome Wide Association Studies (GWAS), several affected and control animals that are related to each other are needed to obtain a definitive result (Figure 8).[11]

Finally, how can we approach other diseases that may have an inherited basis but that have an adult onset? For example, how could we approach an investigation into genetic susceptibility to pyometra? For now, that type of study appears out of reach. First it would likely require long term studies (multigenerational pedigrees) just to identify enough clearly defined normal and affected phenotypes within a group of related animals. Secondly, as it would likely be a complex trait, many more animals would be needed than for a trait inherited as a simple Mendelian trait. However, this should not discourage us from compiling information such as accurate diagnoses and pedigrees to lay the foundation for working up a genetic disease.

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Figure 1.

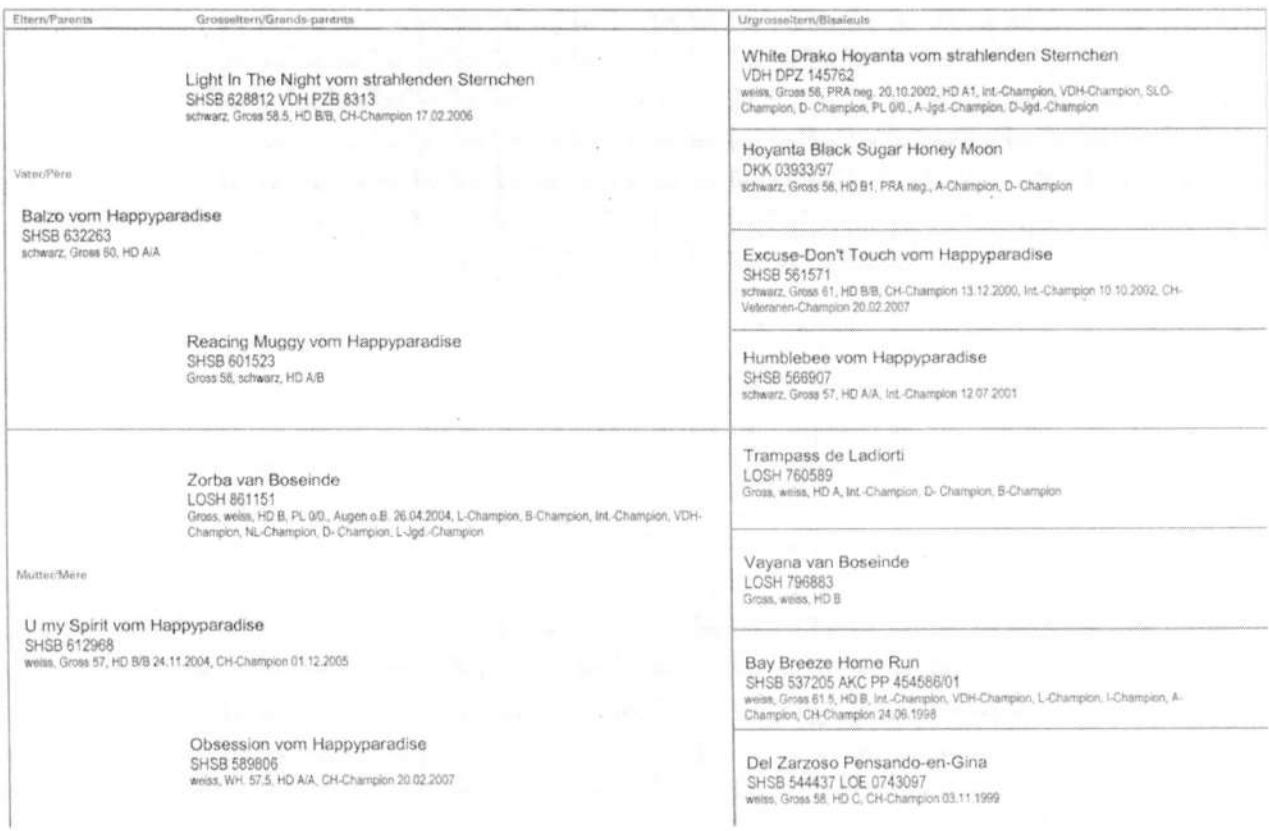


Figure 1. Typical pedigree of a registered dog. Note that the pair listed to the right of any given dog are the parents. Males will always be listed on top and females on the bottom of such pairs.

Figure 2.

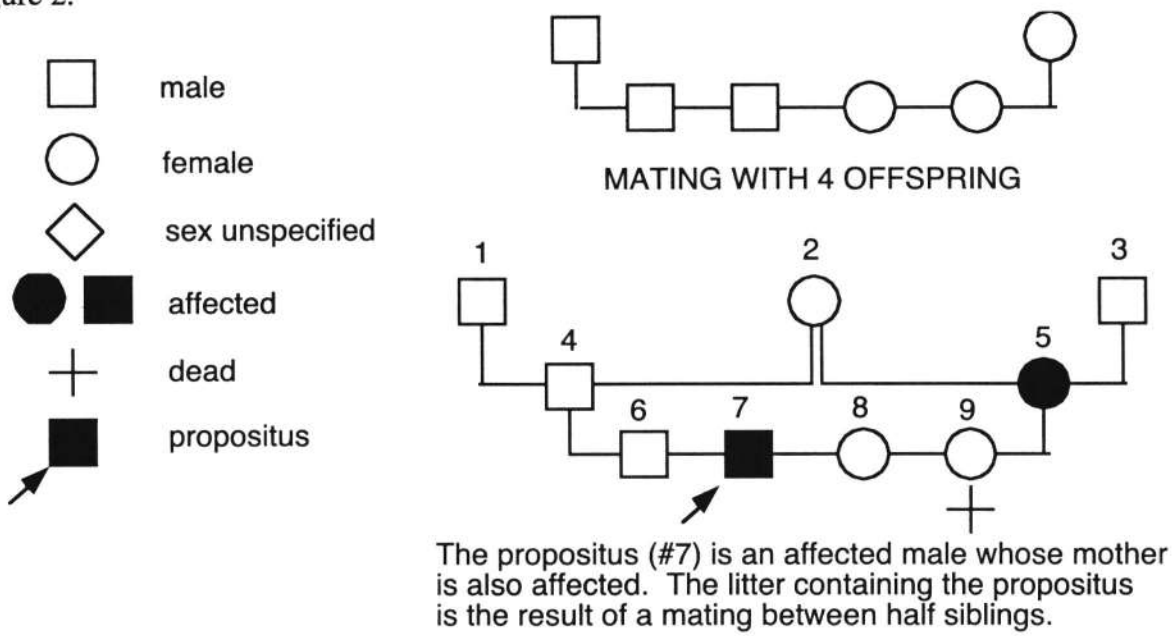
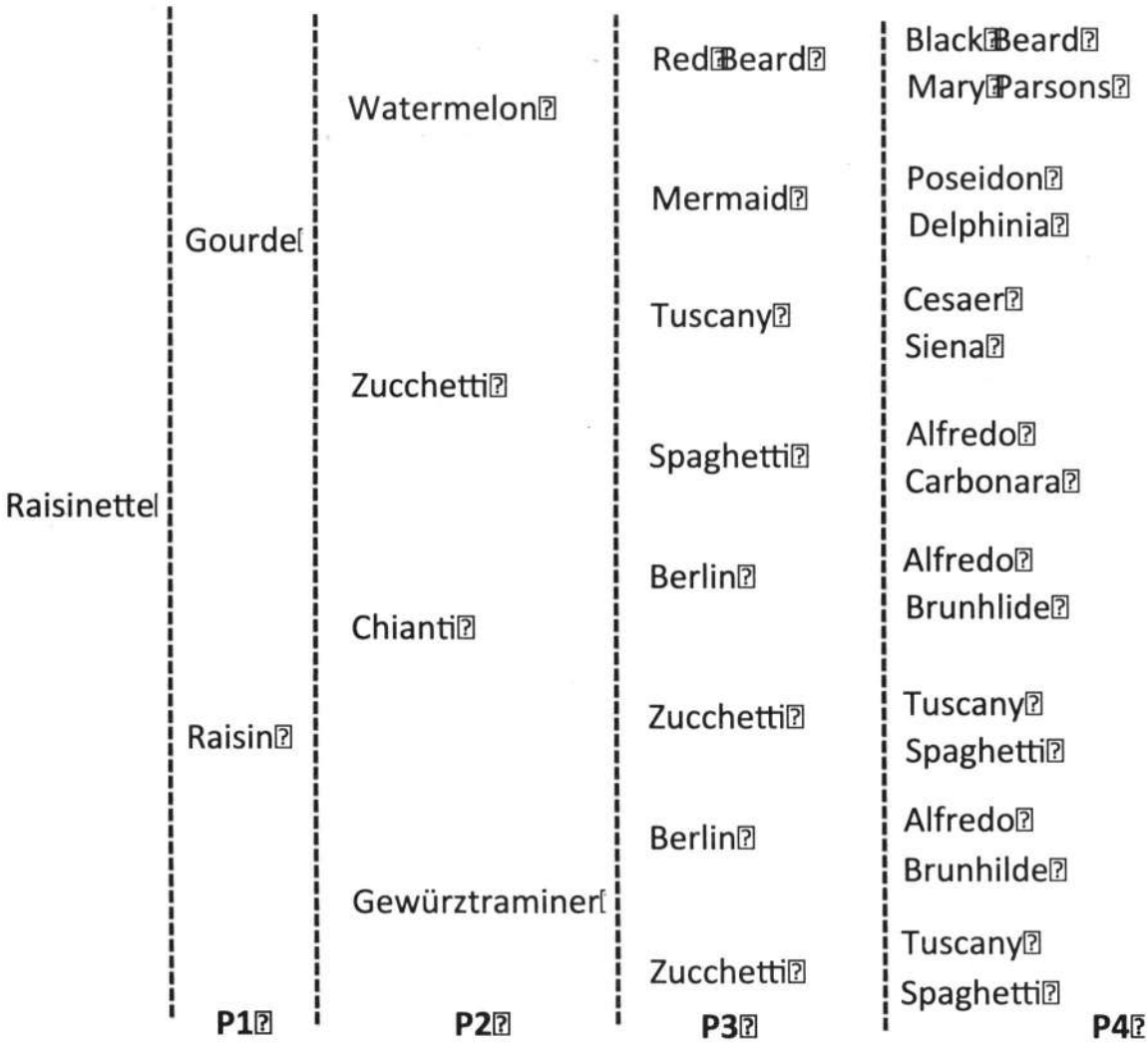


Figure 2. Pedigrees are typically converted for ease of scrutiny by eye. Squares are males, circles females, filled in symbols are affected animals, and empty symbols represent clinically normal animals. Animals that have died can be indicated by placing a cross underneath the individual or drawing a diagonal line across the individual's symbol. Parents are connected by lines and the offspring are indicated on the connecting line.

Figure 3a.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Zucchetti
Berlin
Zucchetti
Black Beard
Mary Parsons
Poseidon
Delphinia
Cesaer
Siena
Alfredo
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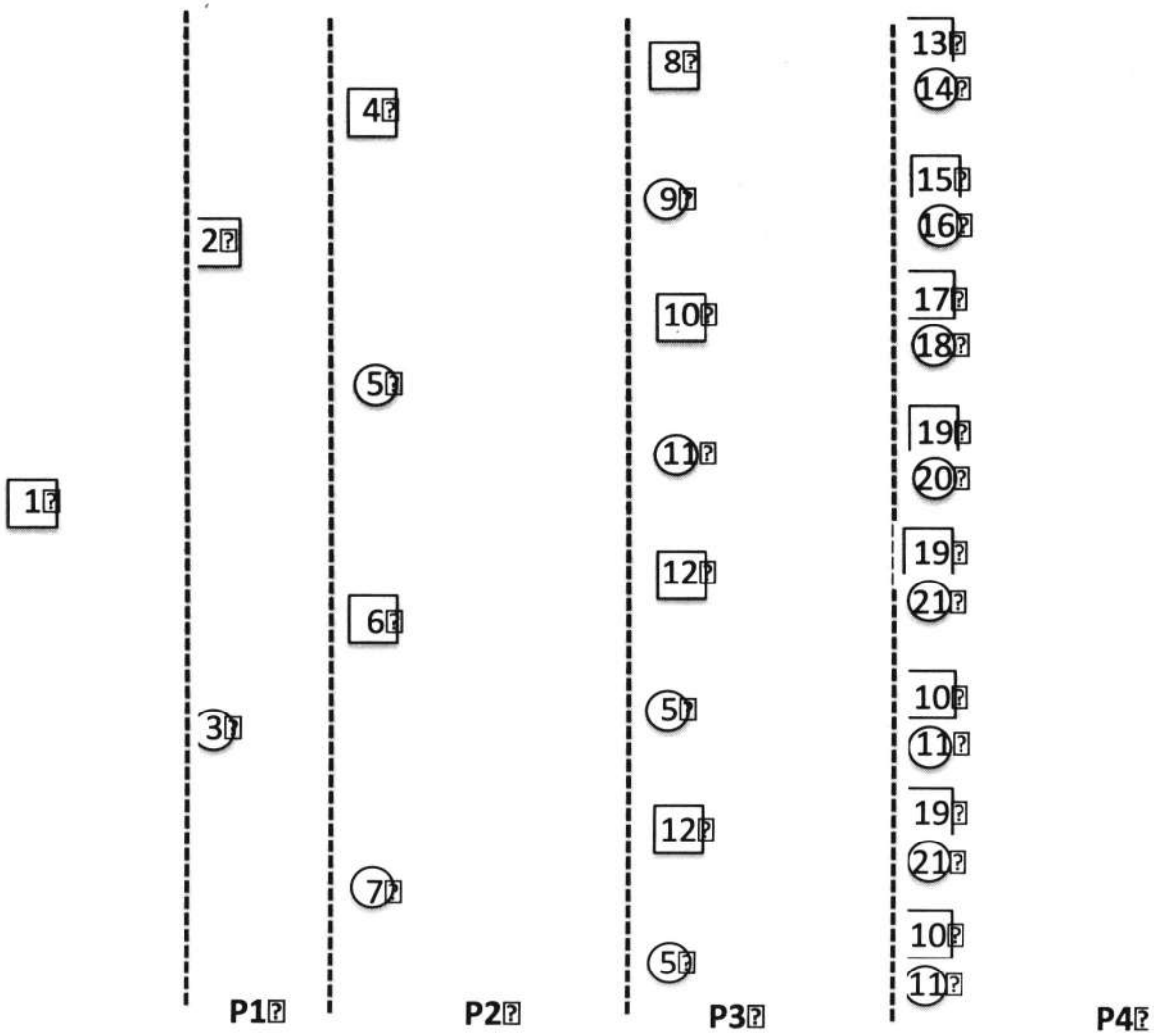
Figure 3b.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P4

Figure 3c.

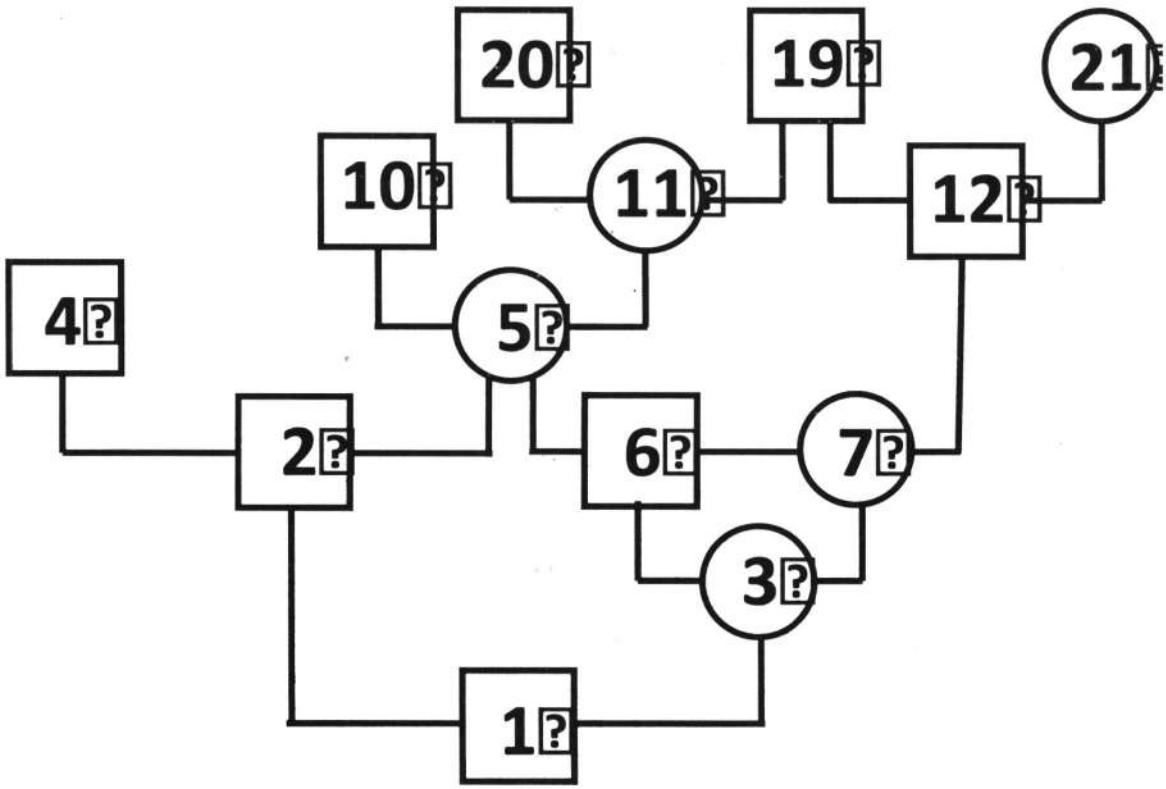

Figure 3. Conversion of a standard pedigree into a graphical pedigree used for analysis. 3a) Standard pedigree. 3b) Names are converted to numbers to quickly identify multiple occurring parents. 3c) Converted pedigree showing only the most important, directly related individuals.

Figure 4a.

1.

	D	**d**
d	Dd	dd
d	Dd	dd

2.

	D	**d**
D	DD	Dd
d	Dd	dd

Figure 4b.

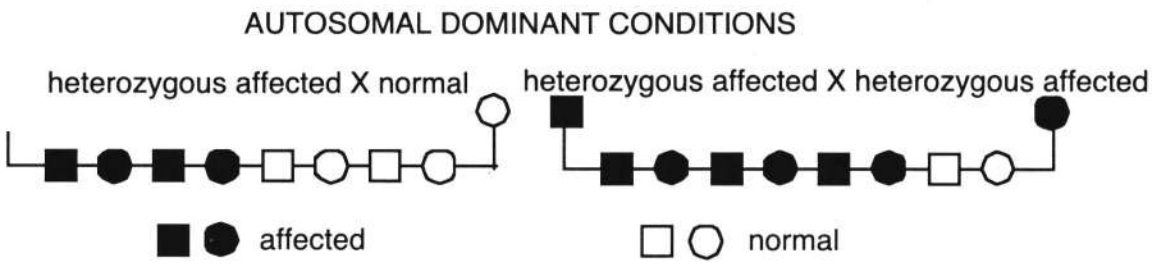

Figure 4. Autosomal dominant inheritance. 4a1) Punnett square demonstrating possible outcomes when pairing affected to normal individuals. 4a2) Punnett square demonstrating possible outcomes when pairing affected to affected individuals. 4b) Pedigrees demonstrating the autosomal dominant mode of inheritance.

Figure 5a.

1.

	R	**r**
R	RR	Rr
R	RR	Rr

2.

	R	**r**
R	RR	Rr
r	Rr	rr

Figure 5b.

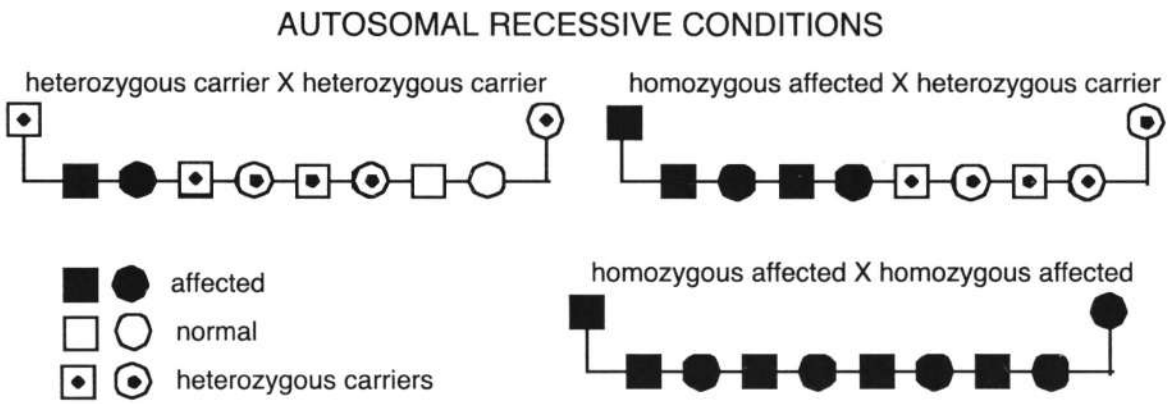

Figure 5. Autosomal recessive inheritance. 5a1) Punnett square demonstrating possible outcomes when pairing carrier to normal individuals. 5a2) Punnett square demonstrating possible outcomes when pairing carrier to carrier individuals. 5b) Pedigrees demonstrating the autosomal recessive mode of inheritance. 5c) Pedigree demonstrating that even if a disease has a straight forward autosomal recessive mode of inheritance, it may not manifest itself until many generations later.

Figure 6a.

1.

	X	X^c
X	XX	XX^c
Y	XY	X^cY

2.

	X	X^c
X^c	XX^c	X^cX^c
Y	XY	X^cY

Figure 6b.

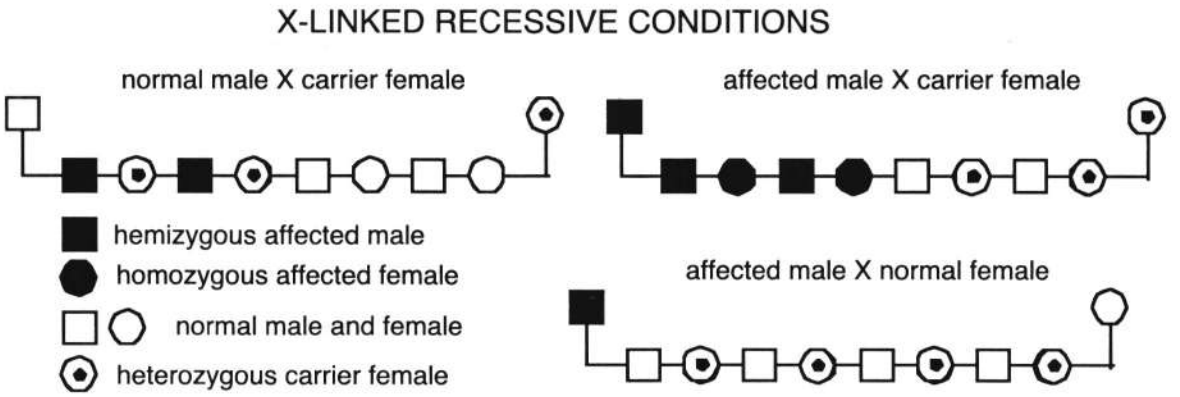

Figure 6. Autosomal dominant inheritance. 4a1) Punnett square demonstrating possible outcomes when pairing a carrier female to a normal male. 4a2) Punnett square demonstrating possible outcomes when pairing an affected male to a carrier female. 4b) Pedigrees demonstrating the X-linked recessive mode of inheritance.

Figure 7.

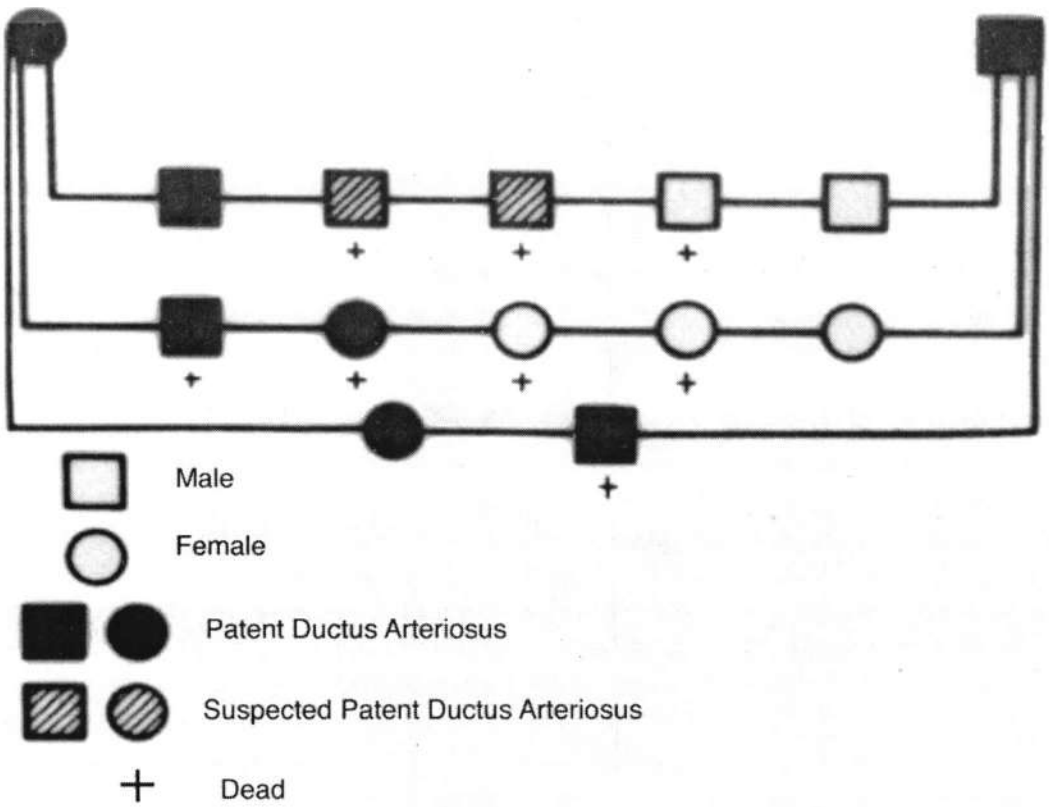

Figure 7. Typical pedigree of a complex genetic disorder in which there is variable expressivity in the offspring, despite the fact that two clearly affected dogs were bred together.

Figure 8.

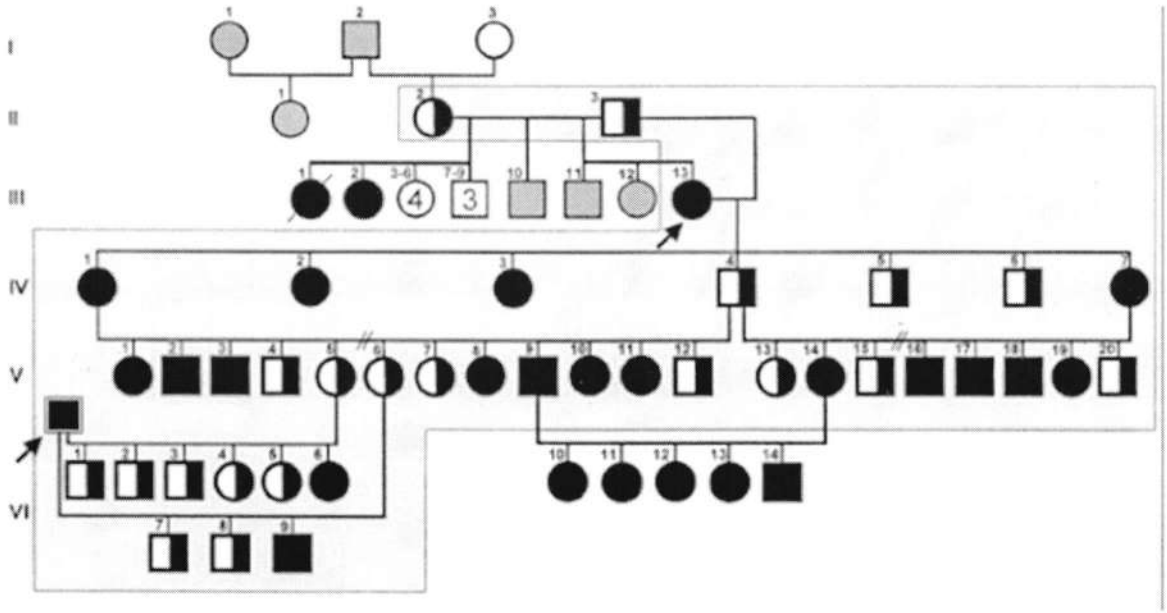

Figure 8. Pedigree developed by breeding affected female (arrow) to carrier father to produce 40 dogs for linkage analysis to discover the basis of centronuclear myopathy in the Labrador retriever.

Bull development (in utero to one year of age)

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Abstract

Age at puberty has a profound effect on the efficiency of cattle production. Since one bull is typically responsible for impregnating multiple females, understanding pubertal changes and factors affecting reproductive development and function in bulls is vitally important. There is a well-documented series of events in development of the testes and establishment of spermatogenesis. Although semen quality and fertility are below minimum standards at puberty (defined as > 50 x 10^6 sperm and > 10% motility), both improve rapidly within a few months thereafter. Nutrition when the bull calf is in utero, and particularly after birth, has a profound effect on reproductive development. In particular, bulls that are very well fed prior to six months of age had earlier puberty, larger testes, and produced more sperm than those with restricted nutrition during this interval. After six months of age, bulls should be fed an adequate diet, as overfeeding will not overcome previous nutritional deficits, and will generally have adverse effects of semen quality and sperm production.

Keywords: Testes, sperm, testicular development, puberty, fertility

Introduction

Age at puberty is a major determinant of beef production efficiency; the ability to use younger bulls for breeding reduces generation intervals and increases genetic gains.[1] Although yearling *Bos taurus* beef bulls are commonly used for natural service in North America, they often have reduced sperm production and poor semen quality, which limit their reproductive performance and delay genetic progress. Furthermore, for bulls which are potential artificial insemination (AI) sires, early puberty enables progeny testing and sire selection to be done at younger ages. Moreover, since one bull can be responsible for breeding anywhere from a few females (via natural service) or hundreds of thousands (via AI), clearly reproductive performance in an individual bull is much more important than in an individual cow. Therefore, understanding pubertal changes and factors affecting sexual development and reproductive function in bulls is vitally important.

Testicular development

Marked hyperplasia of germ cells occurs in utero from days 50 to 80 of pregnancy.[2] In contrast, from days 80 to 200, germ cell multiplication declines, with limited mitosis until the end of the first month of postnatal life.[2] Precursor Sertoli cells increase in number during gestation and in the neonatal period, with mitosis ceasing between six and 10 weeks of age.[3] From zero to eight weeks of age, undifferentiated mesenchymal cells, with high mitotic rates, continuously transform into Leydig cells.[4] There is an early rise in gonadotrophin secretion, primarily luteizing hormone (LH), which stimulates Leydig cells to produce both androstenedione and testosterone.[5] In *Bos taurus* bull calves, the early transient rise in serum LH concentrations occurs from: four to 25 weeks in Hereford and Charolais X,[6] six to 20 weeks in Herefords,[7] and 12 to 16 weeks in Holsteins.[8] In addition, at eight weeks of age, a transient follicle stimulating hormone (FSH) peak stimulates spermatogenesis by inducing germ cell differentiation of gonocytes to prespermatogonia.[9] Increased FSH stimulates Sertoli cell division, maturation, and secretory capacity.[10] However, the testis and hyophysis become increasingly sensitive to LH, due to negative feedback from gonadal steroids, thereby suppressing both the amplitude and frequency of LH surges.[9]

From four to 15 weeks of age, testicular tubular diameters increase from 40 to 80 μm, and germ cells start to proliferate.[2] The prepubertal gonadotropin surge promotes growth and maturation of Leydig cells, which precedes spermatogenesis.[8] Remarkably, Leydig cells (which are derived from mesenchymal cells) account for 17% of testicular volume by 16 weeks. However, from 16 to 30 weeks, a large

proportion of Leydig cells degenerate; those which survive form the population present after puberty.[2] In response to increased LH concentrations during the gonadotropin rise, Leydig cells produce gradually increasing amounts of testosterone, resulting in a slow rise in serum testosterone concentrations from six to 35 weeks. It is noteworthy that testosterone concentrations continue to increase after LH concentrations have returned to near baseline (after the end of the gonadotropin rise). Furthermore, there is a profound increase in serum testosterone concentrations in the weeks prior to puberty, in association with production of mature sperm.[6,7,11]

Testosterone promotes testis growth and spermatogenesis. In that regard, from 20 to 28 weeks, testosterone induced differentiation of supporting cells to become Sertoli cells.[12] Subsequently, the blood-testis barrier is established when junctional complexes form.[11] From 18 to 27 weeks, tubule diameters increase, but they lack a central lumen.[2] In *Bos taurus* bulls, at three, four, five, six, and seven months of age, the most advanced type of germ cell that predominates is the gonocyte, spermatogonia, spermatogonia A, spermatocytes, and mature sperm, respectively.[6,12] Although patterns are similar in *Bos indicus* bulls, development proceeds more slowly, with spermatogonia A, spermatocytes and sperm first detected at approximately 9.5, 11, and 16 months, respectively.[13] The outer seminiferous tubule diameter increases between ten and 45 weeks, whereas inner tubule diameter increases between 30 and 35 weeks.[6] By 24 weeks, LH discharges decline and basal LH declines in Holsteins,[11] but at this age, LH starts to increase to adult concentrations in Hereford bulls.[5,7]

The Sertoli cell, the only somatic cell within the seminiferous tubules, is dependent on FSH and testosterone,[14] and supports germ cells and coordinates their growth and development.[3] The number of Sertoli cells determines the limits of sperm-producing capacity and testis size.[15]

Hormonal control of sexual development in bulls

Sexual development in bulls, which involves maturation of the hypothalamus-pituitary-testes axis, can be divided into three periods: infantile, prepubertal, and pubertal.[16] The infantile period is characterized by low concentrations of gonadotropins and testosterone; it extends from birth to approximately eight to ten weeks of age. A transient increase in gonadotropin secretion occurs from approximately 8-10 to 20-24 weeks; this has been called the "early gonadotropin rise",[6,16] which characterizes the prepubertal period.

Gonadotropin concentrations during the infantile period were low, due to reduced gonadotropin releasing hormone (GnRH) secretion. Although the pituitary was responsive to GnRH in the infantile period, response increased with age.[17,18] Maturation of the hypothalamus increased GnRH pulse secretion, thereby stimulating the transition from the infantile to the prepubertal period of development by enhancing gonadotropin (especially LH) secretion.

During the early gonadotropin rise characteristic of the prepubertal period, there was a characteristic increase in mean LH concentrations from eight weeks to 16-18 weeks of age.[6,16] Mean LH concentrations decreased thereafter and by 20-24 weeks, were comparable to those during the infantile period. The primary impetus for increased LH mean concentrations was a profound increase in LH pulse frequency. The number of LH pulses increased from less than one per day at four weeks to approximately 12 per day (~1 pulse/2 h) at 12-16 weeks.[6] Mean FSH concentrations may or may not increase during the early gonadotropin rise.[5,6,11,16]

Initiation of Leydig cell steroidogenesis was characterized by increased androstenedione secretion, which decreased as these cells completed maturation and begin secreting testosterone, at approximately 16 weeks.[16,17,18] During the first 16 weeks, testosterone concentrations were low and secretion did not necessarily accompany LH pulses. However, after 16 weeks, LH pulses were followed by testosterone pulses and mean testosterone concentrations began to increase. In that regard, the number of testosterone pulses increased from ~ 0.3 to 2.3 pulses/24 hours at four to 16 weeks to 9 to 7.5 pulses/24 hours at 20 weeks. From 24 to 40 weeks, there were ~ 4.5 to 6.8 pulses/24 hours.[19,20] Testosterone concentrations began to increase during the prepubertal period. During the pubertal period, there is accelerated reproductive development from 20 to 24 weeks of age to puberty. During this interval, increased testosterone concentrations were accompanied by rapid testicular deveopment[6,11]

The early gonadotropin rise (especially LH secretion pattern) has an important role in regulating sexual development in bulls. Differences in LH pulse frequency and mean LH concentrations during the prepubertal period were associated with age at puberty in early- and late-maturing Hereford bulls (mean age at puberty 41-42 and 48 weeks, respectively).[5,17] The LH pulse frequency was greater at approximately ten to 20 weeks, and mean LH concentrations increased approximately ten weeks earlier and reached greater maximum concentrations in early- than in late-maturing bulls.[5,17] Furthermore, prolonged treatment with a GnRH agonist in Hereford bulls from 6 to 14 weeks down-regulated GnRH receptors, decreased LH and FSH pulse frequency, pulse amplitude, and mean concentrations at 12 weeks, delayed the peak mean LH concentration from 20 to 24 weeks, and reduced FSH and testosterone concentrations from 14 to 18 weeks.[21] It was noteworthy that these hormonal alterations were associated with delayed puberty (47 and 42 weeks in treated and control bulls, respectively), and reduced PTW and number of germ cells in tubular cross-sections at 50 weeks.[21] Conversely, treatment with GnRH every two hours from four to six weeks of age in Hereford bulls increased LH pulse frequency and mean concentration, mean testosterone concentration, scrotal circumference (SC), PTW, seminiferous tubules diameter, and number of germ and Sertoli cells in tubular cross-sections at 54 weeks of age.[18]

Increasing testosterone secretion and perhaps increasing hypothalamic sensitivity to negative feedback from androgens are likely responsible for the decrease in LH pulse frequency and mean LH concentrations during the pubertal period.[22] Inhibin produced by Sertoli cells may act on the gonadotrophs to limit FSH secretion, since passive immunization with inhibin anti-serum markedly increased FSH concentrations in prepubertal bulls, although LH and testosterone concentrations were not affected.[23] During the pubertal period, testosterone pulse frequency remained unchanged, but pulse amplitude increased with age, with a resulting increase in mean testosterone concentrations until approximately one year.[19,20] Elevated testosterone secretion is essential for increasing the efficiency of spermatogenesis, culminating in puberty.

In bull calves, sexual maturity and increased associated testicular size is related to a rise in concentrations of both testosterone and insulin-like growth factor-1 (IGF-I).[24] In bull calves, plasma leptin increases with age prior to puberty. Both pre- and post-pubertal increases in leptin are associated with increased SC, paired testis weight (PTW), testosterone concentrations and body weight.[24] Leptin may act directly on the testis or through stimulation of gonadotrophin secretion.[25]

Puberty

Puberty in bulls is defined as an ejaculate containing > 50 x 10^6 sperm with > 10% sperm motility.[26] In our experience (unpublished), in ejaculates containing > 50 x 10^6 sperm, the percentage of those sperm that are motile consistently exceeds 10%. *Bos taurus* bulls reach puberty at approximately 8.5-12 mo of age.[26] At puberty, although a bull may be capable of impregnating a female, semen quality is usually poor (high percentage of morphologically abnormal sperm, including many with cytoplasmic droplets).[27] Semen quality improves fairly rapidly, and the proportion of bulls with semen quality meeting minimum standards for a bull breeding soundness evaluation increases up to 12-16 months of age. This has serious implications for breeders that want to sell bulls at young ages (e.g., 12 months), as many of those bulls will not meet minimum standards for semen quality. Furthermore, bulls that reach puberty earlier will have a better chance to be selected as breeding bulls than those with delayed puberty. Better knowledge of factors affecting puberty should improve bull management (including ways to hasten puberty) and the ability to select early-maturing bulls.

Testicular measurements

Testis size is heritable[28] and associated with age at puberty.[29] Breeds with higher SC reach puberty at an earlier age; concurrently, their sisters and daughters also reach puberty earlier and have a higher lifetime pregnancy rate.[30] Since SC is highly correlated with PTW,[12] and PTW is associated with total sperm output, bulls with higher PTW would be expected to impregnate more females. Sertoli cell multiplication ceases at 20 to 25 weeks of age in bulls; therefore, final testis size in the bull may be reliably predicted at this stage.[16] In that regard, testis diameters at five months of age were closely

associated with testis diameters at 12 months;[8] therefore, measurement of SC at weaning can be done to identify bulls with small testes and eliminate them from the pool of potential future sires. Furthermore, testis size at one year of age is generally predictive of testis size at two years of age,[12] although some bulls with delayed puberty (which may result in an extreme frame score due to late closer of the growth plates in long bones), have small to modest sized testes at 1 year of age and relatively large testes at two years. Testis diameter in the transverse plane is greater between 14 and 34 weeks of age and increases faster than the diameter measured in the longitudinal plane, reflecting a change in shape as the testis matures.[7,17]

Differences in testicular measures and reproductive development can be attributed to large differences in genotype. Reproductive development of *Bos indicus* is slower than *Bos taurus* breeds with a longer duration of the prepubertal period in the former.[13,31] *Bos indicus* breeds are also heavier at puberty, with larger SC and paired testis volume than *Bos taurus* breeds,[31] although as yearlings, *Bos indicus* bulls have smaller testes than *Bos taurus*.[32] *Bos taurus* bull calves differing in genotype by 50%, however, do not differ in gonadotrophin concentrations, testosterone concentrations, PTW, or tubule diameters.[6] Earlier maturing *Bos taurus* bulls are heavier than their later maturing counterparts between 16 and 36 weeks of age,[17] or nine and 50 weeks of age.[5] The SC of earlier maturing bulls are also higher from 14 weeks[17] or 26 weeks of age,[5] with greater PTW at castration when compared to late-maturing bulls.[17]

Hormonal indicators of reproductive potential

Circulating concentrations of testosterone and gonadotrophin in prepubertal bulls may indicate sexual maturity and predict age at puberty. Testosterone is positively associated with PTW and tubule diameters in pre- and postpubertal bull calves.[5] Genetic differences in testosterone occur and concentrations are higher in *Bos taurus* than *Bos indicus* breeds.[31]

The reported relationships between LH concentrations and age at puberty are conflicting. Greater pituitary/ hypothalamic activity early in life, may account for higher prepubertal LH concentrations in fast- versus slow-maturing bulls.[5,17] The LH response to LH releasing hormone (LHRH) stimulation, however, was lower in early maturing bulls.[33] Negative correlations between LH and testosterone and paired testis volume have also been reported in bulls between 10 and 70 weeks of age.[34]

Prepubertal concentrations of LH vary with genotype in cattle.[33] The spermatogenic capacity of the adult testis can be influenced by FSH, due to its ability to stimulate Sertoli cell mitosis during testicular development.[10] Prepubertal concentrations of GnRH stimulated FSH are positively correlated with testis diameter and cell ratios in the seminiferous tubules, and basal FSH concentrations are related to number of Sertoli cells per testis.[8] Treatment of bull calves with FSH at four to eight weeks increases SC, hastens the onset of puberty, and enhances spermatogenesis.[14]

Effects of prenatal nutrition on male reproductive development

The hypothalamic-pituitary-adrenal axis is the primary regulator of reproductive function in males and females. In cattle, deficiencies or excesses of metabolites, associated with various diets, are sensed at the level of the hypothalamus and/or the pituitary and may affect gonadotropin release or function. This axis undergoes a series of maturational and functional changes during early fetal life. Nutritional restriction of ewes during gestation reduced LH response to GnRH in fetal[35] and prepubertal lambs,[36] but increased FSH response to GnRH.[37] In ram lambs, prenatal undernutrition, reduces testosterone concentrations, paired testis volume,[38] the number of Sertoli cells per testis,[16] the size of the seminiferous tubules,[37] and delays puberty.[38]

Effects of postnatal nutrition on male reproductive development

Differences in yearling SC due to age of the dam are indirect indications of the effects of nutrition on testicular development. In that regard, SC increased with increasing age of the dam (up to five to nine years of age).[28] The effects on SC are especially marked for bulls raised by two-yr-old dams; they had a 0.7 to 1.3 cm smaller SC than bulls raised by five- to nine-year-old dams.[28] Conversely, SC decreased with increasing age of the dam after ten years of age.[28]

In an older study,[39] Holstein bulls received low, adequate and high nutrition (approximately 60, 100, and 160% of TDN) from one to 80 weeks of age. Relative to the control, bulls fed low nutrition had smaller testes and delayed puberty, whereas those fed high nutrition had larger testes and hastened puberty.

Under field conditions, high-energy post-weaning diets are frequently associated with impaired reproductive function in bulls due to altered testicular thermoregulation. Sperm motility decreased and proportion of sperm defects increased with age in Hereford bulls fed to gain > 1.75 kg/day; these were significantly different from bulls fed to gain approximately 1 kg/day (control) after 76 weeks of age.[40] Even after the high-nutrition diet was changed to a control diet, bulls previously receiving high nutrition continued to have lower semen quality. There was greater deposition of fat around the testicular vascular cone in the scrotal neck in bulls in the high nutrition group; the difference between body and testes temperature was reduced in this group compared to bulls in the control group.[40] This difference was still present after the diets were changed.[40]

In a series of experiments,[41] Angus, Hereford, and Simmental bulls were fed high (80% grain and 20% forage) or medium nutrition from weaning (6-7 months) to 12-24 months of age. In general, bulls receiving high nutrition had greater body weight and backfat, but PTW was not affected by diet. Moreover, bulls receiving high nutrition had lower daily sperm production and epididymal sperm reserves, a greater proportion of sperm abnormalities, and a smaller scrotal surface temperature gradient,[42] perhaps due to decreased heat radiation from the scrotal neck. In another study, bulls fed high-nutrition diets had greater SC than those fed medium-nutrition diets, but PTW was the same.[43] Since scrotal weight was greater in bulls fed high nutrition, perhaps fat deposition in the scrotum increased SC in these bulls. In addition to the deleterious effects of high-energy diets on reproductive function, these diets may also result in abnormal foot growth due to laminitis, as well as abnormal bone and cartilage growth, and may increase the risk of rumenitis, liver abscess, and vesicular adenitis.[44,45]

A series of four experiments was conducted to evaluate the effects of nutrition during calfhood (defined as the period from 10 to 26-30 weeks of age) and peripubertal period (defined as the period from 27-31 to 70-74 weeks) on sexual development and reproductive function in beef bulls).[16] In these studies, increased nutrition during calfhood resulted in a more sustained increase in LH pulse frequency during the early gonadotropin rise and greater testicular development at maturity.[46] Conversely, low nutrition during calfhood suppressed LH secretion during the early gonadotropin rise, delayed puberty and reduced testicular development at maturity.[46] For example, for bulls fed low, medium or high nutrition from ten to 70 weeks of age, age at puberty was (mean ± SEM) 326.9 ± 5.5, 304.7 ± 7.4, and 292.3 ± 4.6 days respectively, and PTW were 523.9 ± 25.8, 552.4 ± 21.1, and 655.2 ± 21.2 grams.[46] It was noteworthy that in bulls fed reduced nutrition prior to approximately 26 weeks of age, and subsequently fed high nutrition, suppression of testicular development and delayed puberty were not overcome.[47] Therefore, it is not possible to compensate for the effects of low nutrition early in life.

When low nutrition was accomplished by restricted feed intake, there was compromise of both hypothalamic and pituitary function, with LH secretion most severely affected.[16] Based on temporal associations between LH secretion patterns and circulating IGF-I concentrations, the latter was regarded as a possible signal to the central "metabolic sensor" involved in translating body nutritional status to the GnRH pulse generator.[16] Nutrition also affected testicular steroidogenesis (testosterone concentrations), indicating effects on the number or function of Leydig cells, or both.[16] Age-related increases in physiological and GnRH-stimulated circulating testosterone concentrations were hastened in bulls receiving high nutrition and delayed in bulls receiving low nutrition; these effects were probably mediated by both LH secretion and IGF-I concentrations.[16] Circulating leptin and insulin apparently have only permissive roles on GnRH secretion, but may enhance testicular development. Growth hormone concentrations decreased concomitantly with increasing IGF-I concentrations during sexual development in bulls, suggesting the testes could contribute considerable amounts of circulating IGF-I. In conclusion, management strategies to optimize reproductive function in bulls should focus on increasing nutrition during calfhood.[16]

Sexual development and reproductive function were studied from six to 16 months of age in 22 Angus Charolais and 17 Angus bulls.[48] Associations of average daily gain and body weight with ages at puberty and at maturity (satisfactory semen quality), SC, paired testis volume, and PTW, testicular vascular cone diameter and fat thickness, scrotal temperature, sperm production and morphology, and testicular histology, were determined. There were no significant correlations between cumulative average daily gain and any of the end points investigated.[48] Body weight at various ages was negatively correlated with ages at puberty and maturity in Angus Charolais bulls, positively correlated with PTW in Angus Charolais and Angus bulls, and positively correlated with seminiferous tubule volume in Angus bulls ($P < 0.05$). Semen quality improved gradually with age and the interval between puberty and maturity (mean $\pm$ SD; 309.4 $\pm$ 29.7 and 357 $\pm$ 42 days of age) was approximately 50 days.[48] Age, weight, SC, and paired-testes volume were all good predictors of pubertal and mature status, with moderate to high sensitivity and specificity (71.6 to 92.4%). In summary, growth rate between six and 16 mo of age did not affect sexual development and reproductive function in beef bulls. However, greater body weight at various ages was associated with reduced age at puberty and maturity, and with larger testes at 16 months of age.[48] Therefore, improved nutrition might be beneficial, but only when offered before 6 months. Average daily gains of approximately 1 to 1.6 kg/day did not result in excessive fat accumulation in the scrotum, increased scrotal temperature, or reduction in sperm production and semen quality, and could be considered "safe" targets for growing beef bulls.[48]

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Bull BSE and semen analysis for predicting bull fertility

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Summary

There are many factors that affect bull fertility. Although few bulls are completely sterile, in an unselected population, 20 to 40% of bulls may have varying degrees of reduced fertility. Breeding soundness refers to a bull's ability to get cows pregnant. The purpose of a standard breeding soundness evaluation (BSE) is to identify bulls with substantial deficits in fertility, although it does not consistently identify subfertile bulls. In that regard, the use of commercial frozen-thawed semen that meets minimum quality standards can result in pregnancy rates with a substantial range in fertility. Although no single diagnostic test can accurately predict variations in fertility among bulls producing apparently normal semen, a combination of laboratory tests may be predictive of fertility. This review is focused on a standard BSE, with some consideration of laboratory tests that can augment the prediction of bull fertility.

Keywords: Bull breeding soundness evaluation, BSE, bull, fertility, semen

Introduction

The term "breeding soundness" refers to a bull's ability to get cows pregnant. Although few bulls are completely sterile (unable to impregnate a female), unless a bull is capable of getting a reasonable number of cows pregnant during a limited breeding season, he is not an efficient breeder. In an unselected population of bulls, 20 to 40% may have reduced fertility.[1]

High reproductive efficiency is the most economically important factor for success in cow-calf enterprises; it has greater economic impact than growth rate, feed efficiency, or carcass quality. Subfertile bulls delay conception, prolong the calving season, reduce calf weaning weights, and increase the number of cows culled due to failure to become pregnant or conceiving late in the breeding season. With multiple sire breeding groups and low bull-to-female ratios, reproductive performance may be adequate, despite the presence of some bulls with poor breeding performance. However, single-sire mating groups and high bull-to-female ratios increase the importance of fertile bulls.

Traditional BSE

The two general methods of evaluating the reproductive potential of bulls are: 1) breeding a large number of normal, fertile females and assessing pregnancy; or 2) conducting a breeding soundness evaluation. Although a breeding trial is the ultimate test of fertility, it is expensive, particularly if reproductive performance is poor. Therefore, it is strongly recommended to conduct a BSE prior to breeding and eliminate bulls expected to have poor fertility. As no single measurement or criterion is a reliable predictor of fertility, several criteria are usually evaluated.

In 1992 after several years of discussion among members of the Society for Theriogenology, the current standards for evaluating beef bulls for breeding soundness were adopted.[2,3] These standards provide a uniform method of assessing a bull's likelihood of establishing pregnancy in 25 or more healthy, cycling cows in a 65-70 day breeding season. Prolonged (or unlimited) breeding seasons preclude opportunities to optimize labor, health maintenance procedures, and marketing opportunities. Furthermore, there are important animal welfare issues, including cows being repeatedly mounted and bred, increased risk of injury to bulls with excessive breedings, and calves born in environmental conditions that threaten their health and survival.

Breeding soundness evaluation is done for three general purposes; prior to the breeding season, prior to sale, and in cases of poor reproductive performance. A bull judged unsuitable for breeding can cause considerable concern to the owner, as well as criticism of the person conducting the evaluation.

Conversely, failure to identify subfertile bulls can result in poor reproductive performance. Therefore, it is essential that veterinarians have the knowledge and skills required to perform BSEs.

Although a BSE is NOT just a semen examination, the latter is a critical component of a BSE. Furthermore, a BSE does NOT guarantee that a bull is highly fertile, nor does it rank reproductively sound bulls regarding their relative fertility, or ensure that bulls are free of viruses or other infectious agents. However, a breeding soundness evaluation generally DOES identify bulls with undesirable heritable traits or bulls unlikely to achieve a high pregnancy percentage in a limited breeding season.

Breeding soundness includes satisfactory general health, physical soundness, libido, and semen quality. The classification is based on physical evaluation and the bull's ability to meet minimum thresholds for testicular development, sperm motility, and normal sperm morphology. Despite the requirement for adequate libido, it is not routinely evaluated. Therefore, the person using the bull for breeding should be reminded that they have a responsibility to observe bulls to ensure that they are identifying, mounting, and successfully breeding estrous cows.

Physical examination

At the start of a BSE, bull conformation, body condition, and overall physical health should be assessed. It is important to observe the bull moving, as lameness may not be apparent in the restrained bull. He should be of appropriate size for his age, free of obvious disease, and should have adequate muscling and body fat. Feet and legs should be free of defects that limit mobility or mounting. Acute or chronic laminitis, excessively straight rear legs (post-leg), sickle hocks (excessive curvature of the metatarsal joint), twisted claws (corkscrew claw), and interdigital fibromas are relatively common musculoskeletal conditions in bulls. Post-leg, sickle hocks, corkscrew claw and interdigital fibromas predipose the bull to lameness and are potentially heritable; therefore, bulls with these conditions are not recommended for breeding. Since it is essential that a bull have good eyesight, the eyes should be carefully examined.

Scrotum and testes (weaned bulls)

The first selection (and culling) of bulls is generally at weaning (approximately 7 to 10 months of age). Since few calves clearly display abnormal development and conformational traits at this age, culling is usually based on the breeder's assessment of the bull's growth potential. The principal reproductive criteria for selection of bulls at this age is testicular development. Bulls as young as seven months with below average scrotal circumference (SC) are predisposed to have below average SC at one and two years of age.[4] vTherefore, starting as early as seven months, any bull with below average SC should be culled. Simmental, Angus and Zebu-derived bulls (predominantly Santa Gertrudis) must have a minimum SC of 23 cm at 198-291 days of age to have a nearly 100% probability of having a SC $\geq$30 cm by 365 days of age.[4] Other continental breeds, predominantly Charolais, and Polled Hereford bulls require a SC $\geq$26 cm to reach $\geq$30 cm by 365 days of age.[4] If minimum requirements for SC are increased to 32 cm at 365 days of age, an additional 2 to 3 cm are needed at weaning.[4] Coe and Gibson evaluated 264 bulls (from 13 beef breeds); they reported that at 200 days of age, calves with a SC >23 cm had a 95% probability of achieving a SC >34 cm by 365 days of age, whereas calves with a SC <23 cm only had a 54% probability of achieving a SC >34 cm by 365 days.[4]

Culling at weaning minimizes losses associated with maintaining cull bulls or mistakenly entering them in performance test programs. Furthermore, weaned bulls that do not have both testes well descended in the scrotum should be culled, regardless of SC. Although cryptorchidism is not common in bulls, it is considered heritable and the bull should not be used for breeding.

Scrotum and testes (yearling bulls)

Numerous studies of performance station bulls indicate drastic differences in semen quality according to age (Table 1); a few months can make a tremendous difference.[5] Although most producers are unwilling to accept that less than 50% of their bulls will be judged satisfactory, if standards are rigorously applied, many young bulls will not meet the minimum standards, typically due to inadequate

semen quality. Good communication with producers, preferably in advance of performing BSEs, are essential.

Table 1. Percentage of yearling bulls (n=254) of various beef breeds with satisfactory semen quality.[5]

Age (mo ± 15 d)	No. bulls	Mean (range) SC (cm)	Satisfactory semen quality (%)
12	40	33.8 (28.5-39.5)	40
13	100	34.5 (28-41)	55
14	84	34.1 (28-45)	56
15	30	34.9 (27-41)	73

Examination of the scrotum and testes

Measure SC by forcing the testes to the bottom of the scrotum and placing a flexible tape around the largest circumference, with sufficient tension so that the scrotal skin is somewhat compressed under the tape. Avoid artificial increases in SC by excessively forcing the testes to the bottom of the scrotum or by forcing the testes to move apart from each other (for example, by wrapping a hand around one testis, with your finger tips on the midline of the anterior aspect of the scrotum). Evaluate the scrotum for scars or other pathology. The scrotum should have a distinct neck; this allows the testes to move away from the body (essential to cool the testes during hot weather). Bulls with little or no scrotal neck, or with an extremely long scrotal neck (bottom of scrotum is ventral to the metatarsal joint) are generally not recommended for breeding.

The testes should be freely moveable within the scrotum, with ≤10% difference in size between testes. Palpate each testis gently for texture (it should be firm and resilient) as well as deeply, to assess areas of firmness that might indicate granulomas, fibrosis, or calcification. Testes that are excessively soft are consistent with testicular degeneration, whereas excessive firmness along the mediastinum testes is consistent with irreversible testicular damage. There should be no adhesions or accumulation of fluid within the vaginal cavity. Palpate the head, body and tail of the epididymides for completeness and for the presence of pain or granulomas. Palpate the spermatic cord for varicoceles or other abnormalities.

There is a rapid increase in SC around the time of puberty. As SC increases with increasing age, the minimum SC (Table 2) varies according to the age of the bull.[2,3] It is noteworthy that these are **minimum** standards; ideally, bulls should be exceed these standards. Furthermore, some persons use breed-specific minimum standards for SC.

Table 2. Minimum SC in bulls, according to age.[2,3]

Age (mo)	Minimum SC (cm)
≤15	30
15-18	31
18-21	32
21-24	33
>24	34

It is well known that SC is highly correlated with paired testis weight, which is correlated with daily sperm production and semen quality traits. Furthermore, SC is a more accurate predictor of age at puberty than either age or weight, regardless of breed. Bulls with a large SC have half-sib heifers and

daughters that reach puberty earlier and are more fertile.[6] Since the heritability of SC in bulls (from one to two years of age) is approximately 0.5, substantial progress can be made in selecting for this trait.[7]

Examination of the internal reproductive organs

Evaluation of internal reproductive organs is an important aspect of a physical examination. Remove feces from the rectum and identify the urethralis muscle and prostate gland. Bulls rarely develop prostatic disease; the prostate is palpable as a transverse band at the cranial extent of the urethralis muscle (approximately 15 to 20 cm from the anus), along the ventral midline of the pelvis. Immediately cranial and dorsolateral to the prostate are the paired vesicular glands. Normal glands feel resilient and are approximately 2 to 4 cm in diameter and 10 to 15 cm long. Just cranial and medial to the vesicular glands are the paired ampulla; they are the termination of the ductus deferens and store mature sperm ready for ejaculation. Each ampulla is 1 to 1.5 cm in diameter, thick walled and 10 to 12 cm long. The ampullae pass under the prostate to empty into the urethra at the colliculus seminalis.

The internal inguinal rings are palpable openings in the abdominal wall, approximately 15 to 20 cm anterior to the pelvic brim and 5 to 15 cm lateral to the midline. Examine for fat, omentum or intestines entering the ring. Bulls with inguinal rings larger than 5-6 cm may be predisposed to development of inguinal hernia.

The most common abnormality detected on transrectal examination is enlargement, excessive firmness or loss of surface lobulation of the vesicular glands. Vesicular adenitis (vesiculitis) was detected in 0.85–10% of yearling bulls during routine breeding soundness evaluations,[8] but is uncommon (<1%) in older bulls. Although both glands may be affected, it is more likely to be unilateral. In semen smears stained with eosin-nigrosin or eosin-aniline blue, neutrophils with intact membranes appear as white, somewhat irregular structures, approximately three times larger than a sperm head. Positive identification can be made by using a blood stain (e.g., Wright's Giemsa). As few as one neutrophil per three high-power fields (1000 x) suggests inflammation somewhere in the reproductive tract (not necessarily due to vesiculitis).

Examination of the penis, prepuce and sheath

The sheath is an extension of the ventral abdominal skin and should be of appropriate size for the bull. Examine the preputial hairs on the end of the sheath for accumulation of blood or exudates which might indicate penile or preputial injury. The presence of small particles (similar to sand) on the preputial hair may indicate urolithiasis. Palpate the entire sheath and penis for areas of swelling or fibrosis. Palpate the dorsum of the distal bend of the sigmoid flexure for evidence of a penile hematoma.

The penis and prepuce are usually examined during semen collection. Examine closely for lacerations, penile hair rings, persistent frenulum, urethral fistulae, or scarring of the prepuce.

Electroejaculation

The most common method for collection of bull semen is electroejaculation. Commercial machines are designed to electrically stimulate the pelvic genitalia via a rectal probe. This stimulation induces the bull to achieve penile engorgement which progresses to penile extension, erection and ejaculation. The stimulation is delivered in the form of a modified sine wave and begins with small voltage for a duration of two to three seconds, followed by a rest period of up to three seconds. Electroejaculators may be battery operated or require direct connection to line current. The operator may manually control the magnitude of current applied and the frequency and duration of pulses, or they may be programmed to gradually increase the intensity and/or frequency of pulsations leading to ejaculation. There are apparent breed differences in how readily bulls ejaculate with electroejaculation; some breeds are more prone to vocalize during the stimulation procedure.[9]

Rectal probes should be of appropriate diameter for the size of the bull and the stimulating electrodes should be confined to the ventral one-third to one-half of the probe. Avoid probes with circular electrodes, as they will stimulate dorsal and/or dorsolateral pelvic musculature, with obvious discomfort to the bull. Smaller hand-held finger electrodes are also available; the operator introduces the hand

bearing the electrode into the rectum and positions the electrodes over the seminal vesicles, prostate and ampullae to induce the bull to ejaculate. Advantages of these electrodes are that the stimulating current is directed directly to the target tissues, with minimal undesirable stimulation of the other pelvic musculature or nerves. However, these electrodes required a skilled palpator to maintain electrode contact with the pelvic organs during stimulation and ejaculation.

Before beginning electroejaculation, palpate the bull's pelvic genitalia by transrectal palpation. Gently massage the urethralis muscles, the vesicular glands and the ampullae. In that regard, transrectal massage of the ampullae may decrease the duration of stimulation required to collect semen by electroejaculation.[10] Although there is wide variation among bulls, most bulls begin to achieve penile engorgement after eight to 15 electrical stimulations, followed by penile extension and erection. As stimulation increases, three to five jets of clear pre-seminal fluid will be ejaculated, followed by four to eight jets of the opaque, sperm-rich fraction. Most operators collect a minimum of three ml of ejaculate for microscopic evaluation. The volume of the ejaculate and sperm concentration are largely determined by how much prostatic and vesicular gland fluid is collected before collecting the sperm-rich fraction. Occasionally a bull will release urine during the electrostimulation; typically, sperm motility will be absent or negligible and the sample will smell like urine. In the authors' experience (unpublished), withholding water for several hours prior to semen collection apparently reduces the incidence of urine contamination. Furthermore, if urine contamination occurs, we typically attempt a second semen collection (within a few minutes after the first sample is collected), with an accelerated 'ramp up' phase to reach maximum stimulation. Regardless of the method of stimulation used, the operator may wish to grasp the free portion of the penis with a dry surgical sponge in order to hold the penis in extension for thorough examination of the penis and prepuce.

Although electroejaculation is a convenient and reliable method of semen collection, there are frequently concerns that it may have adverse effects on bulls. Pharmaceuticals used to facilitate semen collection by electroejaculation or to decrease pain associated with electroejaculation have generally not been efficacious enough to warrant use.[10] Pain associated with electroejaculation may be influenced by operator technique; therefore, operators of electroejaculator equipment must strive to apply electrical stimulation as gently as possible.[10] Although a significant increase in vocalization and plasma cortisol and progesterone concentrations in bulls following electroejaculation was attributed to acute stress,[11] the lack of a difference in plasma concentrations of substance P after electroejaculation was interpreted as a lack of pain associated with nociception.[11]

Alternative methods of semen collection

The ampullae are the dilated termination of the ductus deferens and serve as a reservoir for the next ejaculate. Massage of the ampullae, prostate (with occasional massage of the urethra; massage of the seminal vesicles is not essential) will cause stored semen to be released from the ampullae, resulting in emission (dripping) of semen from the urethra.[12] Disadvantages of this method are that a skilled palpator is required, the procedure is tiring for the operator, care must be exercised to avoid rectal irriation, and there can be no assessment of libido, mating ability, erection ability, or ejaculation. Additionally, the penis usually does not extend and the semen sample dribbles down the preputial cavity and off the preputial hairs. It must be emphasized that the sample is an emission, not an ejaculation and consequently, semen samples are often contaminated with bacteria and smegma from the prepuce. Furthermore, transrectal massage was not as efficacious as electroejaculation for obtaining a semen sample.[12]

Although an artificial vagina is the method of choice for collecting semen at an artificial insemination center, it is rarely used in the field. Alternatively, an intravaginal artificial vagina has been recently developed.[13] Finally, semen can be collected from the anterior vagina of an estrual female after breeding; the vagina should be flushed with saline prior to breeding (to minimize mucus).

Ejaculate volume and sperm concentration

Although the total number of sperm in the ejaculate is used to evaluate sperm production in the

stallion, dog and boar, in bulls the capacity to produce sperm is estimated based on SC. In bulls that are routinely collected with an artificial vagina, ejaculate volume and sperm concentration are meaningful; in bulls collected by electroejaculation, they have little relevance. Regardless, the following terms are frequently used to describe sperm concentration (concentrations are 10^6 sperm/ml):

Very good: creamy, grainy (750-1000)
Good: similar to whole milk (400-750)
Fair: similar to skim milk (250-400)
Poor: translucent (<250)

Sperm motility

Motility is reduced by extremes in temperature (typically low temperature but also excessively high temperature), urine, soap, and other contaminants; therefore, considerable effort should be made to protect semen from these influences. A drop of semen should be placed on a clean, dry microscope slide for estimation of motility. Although mass motion may be observed at low power, high power observation under a cover slip is preferred to assess the percentage of progressively motile sperm in the ejaculate. Concentrated samples should be sufficiently diluted with warm, fresh physiologic saline in order to visualize individual sperm under high power. The threshold for Satisfactory Potential Breeder is a minimum of 30% progressively motile sperm.[2,3]

Sperm morphology

Sperm morphology should be evaluated under oil immersion in order to adequately evaluate individual sperm. Prepare a slide by mixing diluted sperm with eosin-nigrosin stain, similar to a blood smear for evaluation with a light microscope. Alternatively, dilute a drop of the ejaculate with 10% neutral buffered formalin and prepare the slide similar to a blood smear for examination with a phase contrast microscope. Count a minimum of 100 sperm cells; when several abnormalities are present, assessing 300 sperm cells will provide a more accurate count.

There are several systems of classifying sperm.[14] The concept of compensable and noncompensable defects is a reasonable approach to interpreting the importance of sperm abnormalities. Compensable abnormalities can be overcome by increasing the dose; for example, sperm with knobbed acrosomes or bent tails would not be able to induce a zona reaction and prevent normal sperm from fertilizing the ovum. In contrast, nuclear vacuoles are considered noncompensable, as regardless of the number of normal sperm present, the probability of the affected sperm fertilizing the ovum is approximately equal to its proportion in the ejaculate.

Regardless of the method used, it is worthwhile to remember that a minimum number of live, normal sperm are required to populate the oviduct. Once in the oviduct, the sperm need normal membrane receptors to bind to the zona pellucida, a normal acrosome and tail to penetrate the zona pellucida, and a normal nucleus for fertilization. As a general rule, fertility will be decreased if there is >30% morphologically abnormal sperm and >20% head defects.[14]

A common issue during breeding soundness evaluation in yearling bulls is the presence of many sperm with proximal cytoplasmic droplets (one of the most frequent sperm abnormalities in young bulls). This abnormality, often associated with immaturity, can also be present in bulls with testicular degeneration. In immature bulls, the percentage of affected sperm usually declines as the bull completes puberty; most bulls will have satisfactory semen quality in the near future. Therefore, some examiners ignore proximal droplets in yearling bulls, resulting in a higher percentage of these bulls classified as Satisfactory Potential Breeders. However, fertilization rates are markedly lower for bulls with at least 30% sperm with proximal cytoplasmic droplets; as the percentage of droplets decreases, fertilization rates increase. Therefore, according to accepted standards, if the yearling bull is physically sound and meets the other minimum requirements, and due to the presence of proximal cytoplasmic droplets there are <70% normal sperm, the bull should be placed in the Deferred category.

Use of ultrasonography for evaluation of reproductive tissues in the bull

The use of ultrasonography for evaluation of the reproductive tract of the bull has been recently reviewed.[15] Although diagnostic ultrasonography is commonly used for examining the reproductive tract of cows, its use in bulls has been much more limited. A typical clinical ultrasonographic examination of bull testes are unlikely to affect semen quality or sperm production.[16] The ultrasonographic anatomy of bull testes and accessory sex glands have been reported. Although testicular echogenicity increased (i.e. the parenchyma appeared more white) as a bull approached puberty, echogenicity was not superior to scrotal circumference as a predictor of puberty.[17] Ultrasonography can be used to detect and characterize pathology of the testes and accessory glands. It is noteworthy that areas of increased echogenicity (testicular fibrosis) are common, especially in young bulls, but are not associated with decreased semen quality (e.g., proportion of morphologically normal sperm).[15] Neither visual evaluation nor computerized pixel analysis of testicular ultrasonic echotexture were consistently predictive of semen quality in bulls.[15] Therefore, it was concluded that the primary clinical use of ultrasonography for assessment of reproductive function in the bull was characterization of grossly detectable lesions in the testes and scrotum.[15]

Infrared thermography for evaluation of the scrotum and testes

Bovine testes must be 4 to 5°C below body-core temperature (38°C) for normal spermatogenesis.[18] Infrared thermography of the bull scrotum, a non-invasive means of assessing scrotal surface temperature,[19,20] has been used as both a research and clinical tool. A bull is restrained and an infrared thermography camera is held behind the bull and captures the infrared radiation coming from the scrotal surface. The resulting image is composed of a series of pixels, with each pixel having a temperature. The image can be displayed, with colors indicating temperatures, or pixel analysis can be done to objectively characterize temperatures. In one study, infrared thermography was used to assess scrotal surface temperature of 73 yearling beef bulls.[21] Of those bulls, 51, 27 and 22% had a normal, questionable, and abnormal scrotal surface temperature pattern, respectively. Thirty of those bulls, all of which were designated as breeding sound (based on a standard BSE), were each exposed to approximately 18 heifers during a 45-d pasture breeding period (single-sire mating). Pregnancy rate was lower ($P < .01$) for bulls with abnormal scrotal temperature patterns (68%, $n = 8$) than for bulls with normal (83%, $n = 13$) and questionable temperature patterns (85%, $n = 9$).[21] Therefore, it was concluded that infrared thermography has potential for predicting bull fertility. Although the cost of infrared thermography cameras previously restricted their use, cameras satisfactory for imaging bull scrota now cost less < $5 000 (www.flir.com).

Classification for breeding soundness

Following evaluation according to the criteria described above, bulls are classified according to their suitability for breeding on the day of evaluation. Bulls that have a sound confirmation, free of ocular and musculoskeletal defects and that produce at least 70% morphologically sperm, with at least 30% progressively motile sperm, are classified as Satisfactory Potential Breeders.[2,3] Bulls that do not meet these criteria are placed in one of two categories. Those bulls with temporary conditions which are likely to resolve and allow the bull to meet the above thresholds are placed in the category of Classification Deferred. Bulls in this category are usually juvenile, have an injury or lameness that is likely to resolve or suffer temporary testicular degeneration due to hot weather. If this classification is used, the veterinarian should recommend a date for re-evaluation of the bull. Bulls with undesirable heritable defects, small SC, debilitating injury or disease, or with permanent testicular degeneration should be classified as an Unsatisfactory Potential Breeder.

Detailed laboratory tests for semen quality

Sperm motility

Computer assisted sperm analysis (CASA) is much more objective than visual appraisal for assessment of sperm motility. Some aspects of sperm motion characteristics assessed by CASA (namely beat cross frequency, linearity, average path velocity, straightness and curvilinear velocity) were significantly correlated with bull fertility,[22] as were average path velocity, total motility, linearly motile sperm, and total number of motile sperm in swim-up preparations.[23] A recent study comparing low- versus high-fertility dairy bulls demonstrated that sperm from high-fertility dairy bulls have a tendency to undergo hyperactivation (a hallmark of capacitation) immediately after thawing. In addition, characteristics of sperm recovered through a sodium hyaluronate swim up medium better reflected the ability of sperm to undergo hyperactivation and reach the site of fertilization, and thus may be more indicative of in vivo fertility.[24]

Sperm pasma membrane viability

A functional sperm plasma membrane is essential for fertilization; therefore, the integrity of the membrane should be assessed. Eosin-nigrosin is a 'live-dead' or vital stain. In that regards, eosin permeates dead or dying sperm (pink to red sperm heads), whereas viable sperm appear white. The nigrosin simply provides a background to make the sperm easier to see. More recently, various fluorescent probes have been used to identify viable versus nonviable sperm. For example a combination of Syber-14 and propidium iodide[25] is often used. In viable cells (with an intact plasma membrane), Syber-14 makes the DNA bright green, whereas propidium iodide penetrates dead and non-viable sperm and stains them red. The proportion of live and dead sperm (green and red, respectively) is assessed either manually (fluorescent microscopy) or by automation (flow cytometry). Shojaei et al[24] reported that there was a greater percentage of viable sperm after thawing and after swim up in high- versus low-fertility dairy bulls, and that these two end points were positively correlated with fertility. Furthermore, the ratio of sperm recovered after swim-up to viable sperm in post-thaw semen was higher in high- low-fertility bulls. In addition, the proportion of moribund sperm (sperm emit green and red fluorescence due to a compromised sperm membrane) expressed as a percentage of live sperm was low in high-fertility bulls and this end point was negatively correlated with fertility.

Brito et al[26] compared several methods for evaluating sperm membrane viability, including eosin/nigrosin, trypan blue, fluorescent probes, and the response of sperm to the exposure of a hypoosmotic solution. In that study, sperm were used for in vitro fertilization, and fertility was assessed on the basis of the proportion of ova that underwent cleavage. Although all of these stains evaluate the physical integrity of sperm membrane, the hypoosmotic swelling test (HOST),[27] which evaluates the functional competence of the sperm membrane, was the only method that contributed to conventional sperm quality tests in predicting the success of in vitro fertilization.[26]

Sperm DNA decondensation

Sperm chromatin integrity is critical for fertilization and ongoing embryo development. Oxidative stress was suggested as a major cause of sperm DNA damage, with reduced preimplantation embryo development and pregnancy rates.[28] Furthermore, increased testicular temperature reduced the stability of sperm DNA[29] and the ability of these sperm to undergo DNA decondensation and pronuclei formation.[30] The sperm cell structure assay (SCSA) uses flow cytometry to determine chromatin integrity, based on resistance to acid denaturation. Sperm are exposed to low pH and stained with acridine orange, which emits green or red fluorescence when it binds to double- (intact) or single-stranded DNA (denatured), respectively. The ratio of red/(red + green) fluorescence measures chromatin denaturation, which is significantly correlated with fertility.[31,32]

Sperm proteins

Heparin binding proteins were proposed as means of predicting fertility differences among bulls producing morphologically normal sperm[33,34] and there are associations between specific seminal plasma proteins and fertility.[35,36] Sperm proteins differed between low- versus high-fertility Nelore (*Bos indicus*) bulls with acceptable sperm.[37] Similarly, accessory gland fluids from high-fertility Holstein bulls had more bovine seminal plasma protein (BSP) 30 kDa and phospholipase A2, whereas osteopontin appeared to improve the ability of epididymal sperm (from low-fertility bulls) to penetrate oocytes in vitro.[38] In a recent study,[39] scrotal insulation was used to induce abnormal spermatogenesis and sperm proteins associated with abnormal spermatogenesis were identified; there was differential expression (between normal and abnormal sperm of the same bull) of the alpha 4 subunit of Na+/K+ATPase, tissue inhibitor of metalloproteinase-2 (TIMP-2), angiotensin converting enzyme (ACE), and hexokinase-1.[39]

Abnormal sperm

Morphologically abnormal sperm failed during gamete interaction or pre-implantation development.[40,41,42] In addition, embryos resulting from fertilization of oocytes by morphologically normal sperm, co-existing in the ejaculate along with abnormal sperm, had reduced developmental competence, suggesting that these sperm were functionally impaired. Therefore, increasing the insemination dose to compensate for infertility due to compensable factors[43] requires further investigation. Data from commercial embryo production units suggested that bulls differ in their ability to produce preimplantation embryos in vivo (Thundathil and Mapletoft, unpublished data). In that regard, damage to sperm DNA due to oxidative stress, chromosome anomalies and environmental effects, including elevated testicular temperature, time of AI relative to estrus, duration of semen storage, duration of sperm-oocyte interaction, age of males, and infectious agents in semen influence embryo quality (reviewed by Chenoweth, 2007).[44]

Models utilizing multiple variables

A seven-variable model (post-thaw total motility, post-thaw sperm with a linear motile pattern, sperm concentration, concentration of motile sperm after swim-up, sperm ZP-binding, cleavage rate of total oocytes, and blastocyst rate of total oocytes) accounted for 84.6% of the variation in non-return rates.[45] However, this approach may not be sensitive enough to discriminate among highly fertile bulls. Similarly, a model with 30 post-thaw sperm characteristics (including cleavage rate) accurately predicted fertility (based on conception and non-return rates) of both high- and low-fertility bulls.[46]

Conclusion

A traditional breeding soundness examination will usually identify bulls that are grossly abnormal. However, variations in fertility among bulls classified as satisfactory suggest that submicroscopical differences may exist in the sperm characteristics of these bulls. Therefore, understanding the molecular basis of these differences in sperm characteristics may enable us to develop laboratory assays to complement traditional breeding soundness evaluation and better predict fertility of bulls.

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Nutritional influences on reproduction: effects of endophyte-infected tall fescue on beef cattle performance

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Introduction

Tall fescue, a cool-season perennial grass, is one of the most commonly grown forages for over 8.5 million cattle in the United States.[1] Cattle suffering from fescue toxicosis experience decreased feed intake and performance, elevated respiration rate and body temperature, rough hair coats and necrosis of the extremities (tail, hooves, and ears) due to loss of circulation.[2] Endocrine and reproductive effects of fescue toxicosis in cattle include decreased calving rate[3] and pregnancy rates,[4-6] reduced circulating concentrations of hormones such as cortisol, prolactin (PRL),[7] progesterone (P_4),[8,9] and LH.[3,8] This toxicosis results in estimated losses to the United States beef industry of $609 million annually due to lowered conception rates and depressed body weight gains.[1,2]

We have made considerable progress in "identifying the window" in timing of reproductive loss associated with grazing infected tall fescue. We also know that addition of clover to our pastures will help reproductive performance in cattle, as does the addition of supplemental feeding of grain (etc.). This supplementation with clovers and grain is thought to have a "diluting" effect on the toxic component of tall fescue grass. In technical terms, the assumed "bad" component of tall fescue infected with the endophyte, *Neotyphodium coenophialum,* is an alkaloid known as "ergovaline" (produce by the endophyte) that negatively affects performance of the animal but plays a beneficial role on the hardiness of the grass.[10-13] However, recent research by Hill et al. indicates that transport of the ergopeptine alkaloid "ergovaline" across ruminal gastric tissue is low as compared to the simple ergoline alkaloids such as lysergic acid; thus, suggesting other alkaloids may play a larger role in tall fescue toxicosis.[14]

So with all this said, the tall fescue research team at the University of Tennessee has focused their research attention on determining "how" and "when" the grazing of endophyte-infected tall fescue (E+) affects reproduction in cows and bulls. We performed these studies by either grazing tall fescue pastures (E+ or MaxQ, non-toxic endophyte, NTE) or by using a synthetic compound called ergotamine tartrate (referred to as ERGOT) to simulate the negative effects of ergovaline since ERGOT was commercially available, presented the same signs of tall fescue toxicosis, and we could control the nutritional status of the animal (thus removing nutrition from the equation as it relates to reproduction). Now to the reproduction part, we first wanted to know **when** consumption of endophyte-infected tall fescue had a negative effect on reproduction. There are several different periods of concern when looking at reproduction in a beef cattle setting, so we broke these time periods into different stages beginning with (1) the effect on the bull, (2) late pregnancy losses in the cow, (3) losses due to hormonal changes before estrus (heat), and (4) embryonic or uterine losses immediately following estrus.

Effects on the bull

Few studies have focused on the beef bull, as related to fescue toxicosis, which were conducted with sufficient numbers or replicates to draw conclusions. Studies in mice[15] and dairy bulls[16] had suggested a detrimental effect (mice) or no effect (Holstein bull calves) when consuming tall fescue seed or hay, respectively. Alamer and Erickson reported that yearling beef bulls grazing E+ tall fescue contained fewer Sertoli cells, suggesting impaired testicular function.[17]

Analysis of data from a three-year project performed at Highland Rim Research and Education Center with 96 yearlings beef (Angus and Gelbvieh) bulls presented some interesting findings. Year 1[18] of the experiment involved yearling bulls receiving a control diet of corn silage supplemented with soybean meal (n=8) or a treated diet consisting of corn silage supplemented with soybean meal and ergotamine tartrate (n=8) for a period of 224 days (November through June). This study was performed to control for the detrimental effect of tall fescue consumption on nutrition or weight loss. Again, feeding of ERGOT allows for us to control for the "nutrition factor" by regulating feed consumption and enables us to focus on the effects of the alkaloid on male fertility. Years 2 and 3[19] utilized two sets of yearling

beef bulls to evaluate the effect of actually grazing (experimental period of 224 days) non-toxic tall fescue (NTE; MaxQ; n=10/year) or endophyte-infected (E+) tall fescue with (n=10/year) or without clover (n=20/year). Body weights, blood samples, forage samples and rectal temperatures were collected every two weeks. Every 60 days, scrotal circumference was recorded and semen collected for evaluation of motility and morphology. Testicular core temperatures were measured immediately before semen collection at the beginning of May and the end of June each year. Semen was extended immediately following collection and returned to the laboratory for evaluation through our in vitro fertilization program to determine fertilization potential and subsequent embryo development.

In brief, bulls consuming the diet supplemented with ergotamine tartrate had similar weight gains to control bulls as desired for the study. Scrotal circumference and semen motility and morphology were similar between treated and control bulls but fertilization potential (cleavage) was reduced (Table 1) in ERGOT bulls compared to controls. Subsequent development of embryos that cleaved was similar between treatments. However, testicular core temperatures were reduced in ERGOT bulls (Figure 1) compared to controls even though rectal temperatures were elevated suggesting a vasoconstrictive effect of consuming ergotamine tartrate on the testis.

During Years 2 and 3, yearling beef bulls grazing E+ pastures without clover performed poorly (average daily gain) but no differences were noted in scrotal development or semen quality (as evaluated during a breeding soundness examination) in May and late June compared to bulls grazing NTE (MaxQ) pastures. However, the fertilization ability or potential (ability to cleave) of the semen was reduced (Table 2) in bulls grazing E+ pastures without clover compared to NTE pastures. Again, rectal temperatures were elevated but with a reduction in testicular core temperatures (as measured by thermography) in bulls grazing E+ tall fescue pastures. Addition of clovers to the E+ pastures improved gain performance but fertilization potential of semen was not determined. Additional studies have just been completed investigating pregnancy rates associated with artificial insemination of heifers and penetration rate of semen from bulls grazing either E+ or NTE pastures.

Table 1. Ability of sperm collected from bulls fed a control or ergotamine tartrate-supplemented diet to fertilize bovine oocytes.[18]

TRT	REP (n)	COC (n)	PZ (n)	Cleav (%)	Blast (%)
CON	2	200	169	69.2±3.3[a]	22.2±3.1
ERGOT	2	200	143	51.1±3.3[b]	22.0±3.1
P-value				0.001	0.96
Lab Con	2	100	86	74.4	43.3

[a, b] Least squares means differ within a column
Reps: total number of replications per bull (Replicate 1, May 5th; Replicate 2, June 28th)
COC: cumulus oocyte complexes
Cleav: number of putative zygotes cleaved
Blast: blastocyst; percentage of cleaved embryos developing to blastocyst

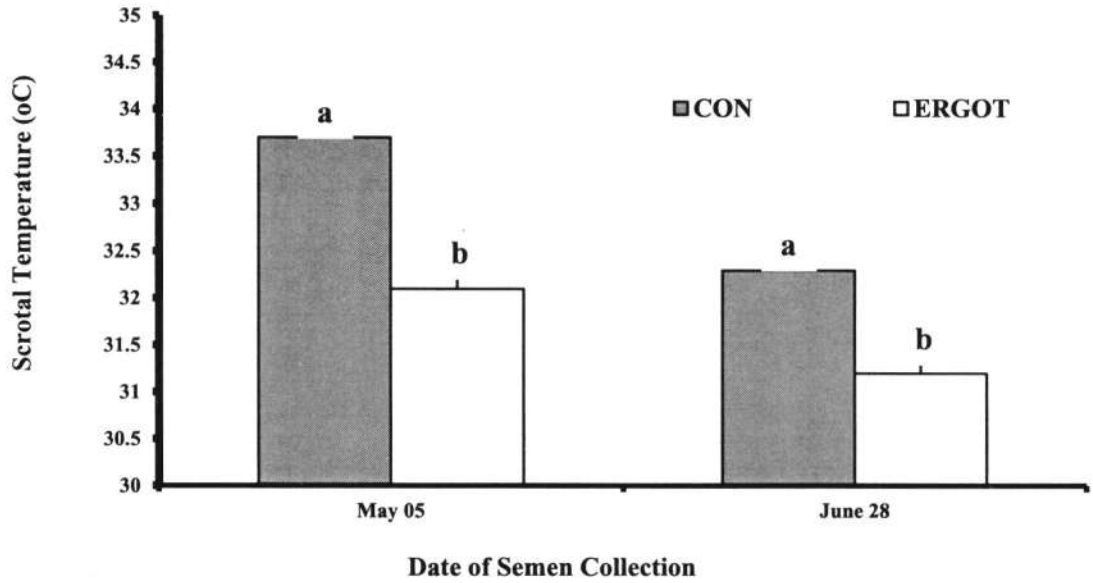

Figure 1. Scrotal thermography measured at the time of semen co llection on May 5th and June 28th. Scrotal temperatures recorded immediately before semen collections were lower in bulls administered ergotamine tartrate (ERGOT) compared to control (CON) animals ([a, b] Least squares means differ within treatments; $P<0.05$).[18]

Recent data indicate that the reduction in cleavage is associated with reduced penetration rate of spermatozoa into the oocyte as well as altered intracellular Ca^{2+} attributes of the fertilized ovum.[20] Also, semen collected from bulls grazing E+ tall fescue pastures have reduced post-thaw motility and a rapid decline in motility with a 3-hour post thaw stress test,[20] suggesting a more fragile spermatozoa in these bulls grazing E+ tall fescue. Potential studies of interest include evaluation of tall fescue effects on bulls that have been removed from these pastures for a period of time then re-introduced back on tall fescue pastures during the breeding period and causes for altered penetration rates.

Table 2. Ability of sperm collected from bulls grazing tall fescue pastures to fertilize bovine oocytes.[19]

Variables	NTE	E+	Lab Control
Rep (n/yr)	2	2	2
COC (n)	850	873	278
Cleav (%)	84 ± 2.4^a	73.5 ± 3.1^b	81 ± 3.8
Blast (%)	30.1 ± 4.7	32.4 ± 5.5	30 ± 5.5
Nuclei (#)	76.5 ± 4.5	72.9 ± 4.8	73.6 ± 5.2

[a, b] Least squares means differ within a row, $P < 0.05$
COC: cumulus oocyte complexes
Cleav: number of putative zygotes cleaved
Blast: blastocyst; percentage of cleaved embryos developing to blastocyst
Nuclei #: total number of cells in blastocyst after fixation and Hoechst staining

Effects on the cow

On the female side, it was observed that late pregnancy losses *were not occurring* at a higher rate in cows and heifers grazing E+ tall fescue as compared to animals grazing endophyte-free pastures. Heifers grazing infected tall fescue that were confirmed 30 days pregnant by ultrasonography stayed

pregnant at an acceptable rate AND that the reproductive loss had already occurred by 30 days.[21] Thus, we deleted *late pregnancy losses* as a concern in cows grazing endophyte-infected tall fescue. Similar results have been reported by several laboratories suggesting that reproductive losses occur early during pregnancy.

Next, we wanted to determine if changes in reproductive hormones or the follicle that produced the egg (oocyte) that would result in pregnancy differed in animals receiving ergotamine tartrate (ERGOT) to simulate fescue toxicosis.[6] In brief, pregnancy rates determined at 30 days postbreeding were reduced in cattle receiving ERGOT without any changes in reproductive hormones (Figure 2) or alterations in growth of the ovulatory follicle. Contrasting results have been reported concerning follicular development and alterations in reproductive hormones[22-24] with others in agreement.[25] Thus, in our laboratory with ergotamine tartrate, effects of fescue toxicosis were evident on pregnancy but not on any reproductive events that occurred immediately before breeding.

Next, we asked the question of whether the uterus was capable of maintaining a pregnancy when a good embryo was transferred into the reproductive tract on day 7 after estrus. In short, was fescue toxicosis causing a problem within the uterus? Rahe et al.[26] suggested that losses were occurring after d 7 of pregnancy (following embryo transfer) but left several unanswered questions. We observed that pregnancy rates were acceptable following transfer of frozen-thawed embryos (collected from cows not exposed to E+ tall fescue) in both groups of heifers that served as either controls or those receiving ERGOT.[27] Even though rectal temperatures were elevated, pregnancy rates were 50% in ERGOT heifers following embryo transfer. Thus, we deleted the *uterine environment after day 7* as a factor in reduced pregnancy rates during fescue toxicosis in heifers receiving ergotamine tartrate.

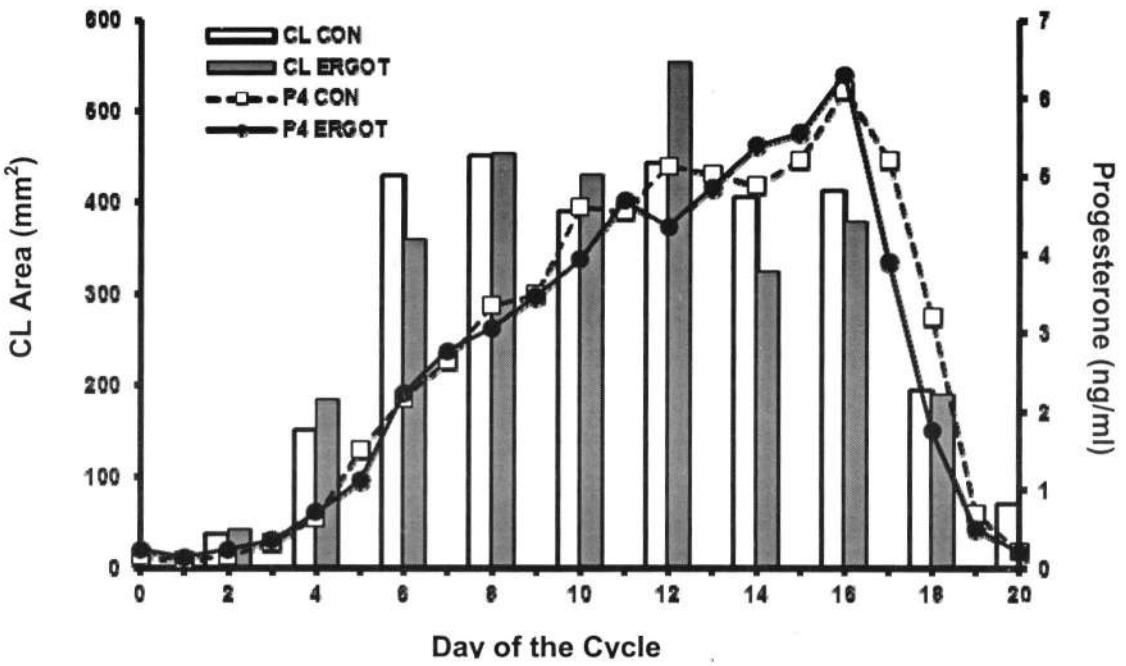

Figure 2. Area of the corpus luteum overlayed with progesterone concentrations during the ultrasonography period.[5]

So when is fescue toxicosis affecting reproduction in the cow? The next experiment provided us a "smaller time frame". Heifers were allotted into their respective treatment groups: receiving or not receiving ERGOT to simulate fescue toxicosis.[27] Estrus was synchronized and heifers (non-follicle stimulating hormone stimulated) inseminated with semen from the same bull.

Seven days later, single embryo recoveries were performed and embryos graded (evaluated) for quality and development. Embryos from heifers receiving ERGOT were reduced in quality and development (Figure 3). In other words, they were poor compared to our control embryos. Furthermore, recovery rate tended to be reduced in heifers supplemented with ergotamine tartrate.

Summary

So what does this all mean? Briefly, fescue toxicosis affects either the growing oocytes (egg) or the early embryo while still in the oviduct on the female side. Add in the effects of tall fescue on the sperm and we can understand why fertility is reduced. Throw in elevated temperatures during the summer months (June and July) with little (or no) clover and we could see a "reproductive wreck". What we can suggest in terms of management is that we can remove cows from fescue for 30 days before and after breeding and see no effect on pregnancy rates (is this practical?). More practical would be to have your cows calve early (if spring calving) and get them exposed to the bull before the hot summer months occur (the same with your heifers). We intend to perform additional studies to determine if we can manage our females differently around the time of breeding (additional feeding, change pasture locations and types, etc.) to improve pregnancy rates when grazing infected tall fescue.

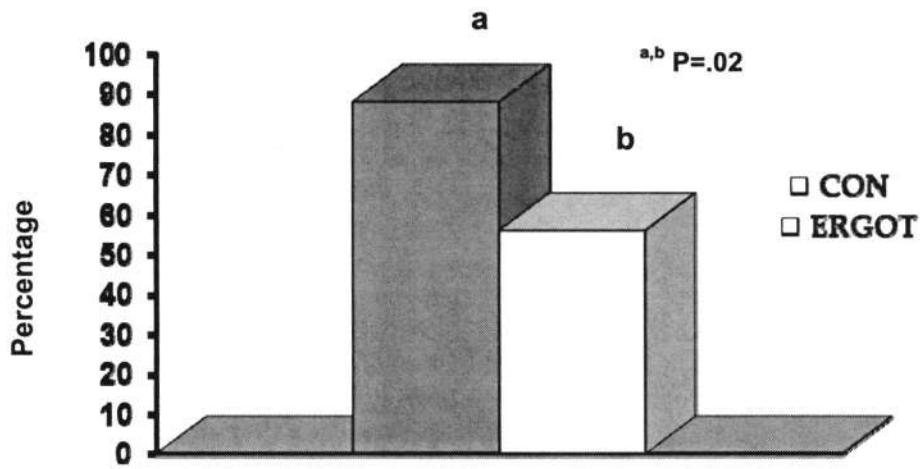

Figure 3. Development (percentage of embryos that had atttained the compacted morula or blastocyst stage) of embryos recovered on day 7 after estrus in heifers consuming ergotamine tartrate to simulate tall fescue toxicosis or serving as controls.[27]

Another factor to keep in mind would include the possibility that you have selected, over time, animals that will tolerate E+ tall fescue. You have culled those heifers that did not grow well. You have sold those cows that would not get pregnant. Has your herd adapted to grazing tall fescue? What about the bull? Will a bull that has not been exposed to tall fescue in his lifetime work on your farm? All the animals used in the above studies had been born and raised on the farm where the research was performed and consumed E+ tall fescue (with clover) their entire life. So have your animals been selected for adaptation? I'll leave that question for the genetic researchers!

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Pain assessment in cattle

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Introduction

The 21st century consumer is wealthier but also more detached from production agriculture than any in history. Therefore, animal welfare concerns are becoming an important issue to our clients and customers. We recognize that pain is an inevitable consequence of many routine animal husbandry procedures in farm animals. However, how can we assess if cattle are in pain and if our analgesic interventions are working? In this session we will examine novel approaches to measuring pain and analgesic drug efficacy in cattle. We will discuss a practical sub-anesthetic/ analgesic drug combination you can use to take the edge off the fractious cases you encounter.

Nociception is an inevitable consequence of many routine management procedures in farm animals. Castration is considered one of the most stressful experiences for livestock by the American Veterinary Medical Association (AVMA) [1] and is performed on approximately 15 million calves in the U.S. annually.[2] Dehorning and castration are especially significant in terms of animal welfare because preemptive analgesia can be applied in advance of the painful stimulus, thereby preventing sensitization of the nervous system to subsequent stimuli that could amplify pain. The AVMA "supports the use of procedures that reduce or eliminate the pain of dehorning and castrating of cattle" [3] and proposes that "available methods of minimizing pain and stress include application of local anesthesia and the administration of analgesics."[1] In spite of this, a recent survey of 184 bovine veterinarians conducted by our research group found that only one in five U.S. veterinarians use anesthesia or analgesics at the time of castration. One reason for this discrepancy is the lack of objective methodology to quantify the most effective pain mitigation strategies.

Keywords: Pain assessment, pain management, substance P, meloxicam, gabapentin

Castration

It is remarkable that although administration of local anesthesia prior to castration and dehorning is legislated in several European countries there are currently no analgesic drugs specifically approved for pain relief in livestock by the U.S. Food and Drug Administration (FDA).[3-6] FDA Guidance Document 123 for the development of effectiveness data for non-steroidal anti-inflammatory drugs (NSAIDs) states that "validated methods of pain assessment must be used in order for a drug to be indicated for pain relief in the target species."[7] The identification and validation of robust, repeatable pain measurements is therefore fundamental for the development and approval of effective analgesic drug regimens for use in livestock. Research to address our limited knowledge in this area is therefore essential to formulating science-based recommendations.

In practical terms, resistance to requiring injectable analgesia for routine castration and dehorning is largely based on time and logistical issues. However, a recent study evaluating novel methods of analgesia for tail docking in lambs demonstrated that castrating by banding in 1-2 day old lambs required an average of 28 seconds without analgesia and 68 seconds when an injectable local anesthetic was administered by jet-injector. [8] While the U.S. has not followed other countries in legislating the use of local anesthesia during castration and dehorning, it is appropriate for the veterinary profession to pursue practical, rapid, and effective methods for the relief of pain related to these procedures. The authors are aware of practicing veterinarians that have adopted local anesthesia regimens for these procedures.

Candidate methods to assess pain associated with castration

The literature pertaining to behavioral response associated with castration has been summarized in an excellent review by Stafford and Mellor.[11] The salient points pertaining to this discussion are summarized below.

Behavior. Assessment of individual animal behavioral changes in response to pain is highly subjective. Escape behaviors demonstrated at castration but not seen afterwards may reflect a pain response[9] or a desire to escape confinement.[10,11] Studies have reported that surgically castrated calves struggle and kick during the operation but calves castrated with rubber rings are quieter.[12] Macauley and others[13] reported that calves castrated surgically moved around much less than control calves or calves castrated using a Burdizzo emasculatome. Two days following castration, the surgical and Burdizzo emasculatome castrated calves were observed to be less active than control calves. Robertson and others[14] found that rubber-ring, Burdizzo emasculatome and surgical castration caused significant behavioral responses indicative of pain during the first three hours after castration. Fisher and others[15] reported that 14 month old bulls castrated surgically stamped their hind feet, swished their tails and grazed less in the afternoon following castration than control bulls and bulls castrated using bands. Behaviors indicative of a painful sensation such as turning the head towards the hindquarters, alternate lifting of the hindlegs, abnormal postures and slow movement of the tail has been reported weeks after rubber-ring castration.[16] The relative level of pain associated with behaviors seen after castration has not been quantified.[17] The characteristics of emotional states such as being fearful, anxious or happy; and other subjective states such as pain sensation and perception are such that they can never be precisely and accurately measured.[18]

Production parameters. Production parameters are often too imprecise to reflect the pain experienced by animals following castration. Furthermore, weight gain following castration may be negatively influenced by a decrease in testosterone following removal of the testes.[19] However, assessment of production parameters is critical if animal well-being research is to have relevance to livestock producers. These assessments may take the form of a cost-benefit analysis or a measure of animal performance. In some studies, Burdizzo emasculatome or surgical castration had no effect on average daily gain (ADG) over a three month period following castration.[20,21] The ADG of 7 week-old calves during the 5 weeks following castration using rubber rings, clamp or surgery have been reported to be lower than non-castrated calves but similar between the different castration methods. Rubber ring and surgical castration were reported to cause a decrease in ADG of 50% and 70% respectively in cattle aged 8 to 9 months.[22] When 8, 9 and 14 month old cattle were castrated surgically or using latex bands, cattle castrated later had poorer growth rates than those castrated at weaning. Cattle castrated with latex bands also had lower growth rates than those castrated surgically during the following 4–8 weeks.[15,19] In a study conducted by Oklahoma State University, 162 bull calves were used to determine the effects of latex banding of the scrotum or surgical castration on growth rate. Bulls that were banded at weaning gained less weight than bulls that were banded or surgically castrated at 2 to 3 months of age.[23] In a second study, 368 bull calves were used in two separate experiments to examine the effect of method of castration on receiving health and performance. In the first experiment latex banding intact males shortly after arrival was found to decrease daily gain by 19% compared with purchasing steers, and by 14.9% compared with surgically castrating intact males shortly after arrival. In the second experiment purchased, castrated males gained 0.58 lbs more and consumed 1.26 lbs more feed per day than intact males surgically castrated shortly after arrival.[24]

Cortisol response. Several studies have evaluated acute cortisol response as a method to determine the extent and duration of distress associated with castration in cattle.[15,17,25-27] Studies reviewed by Stafford and Mellor[11] indicate that surgical and latex band castrations, especially when performed in older cattle, appear to elicit higher plasma cortisol responses that remain above pre-treatment levels for longer periods. Peak cortisol concentration following surgical castration occurs within the first 30 minutes after castration and range from 45 nmol/L following rubber ring castration to 129 nmol/L following surgical castration. The duration of plasma cortisol response above pre-treatment levels typically ranges from 60 minutes following Burdizzo emasculatome castration to 180 minutes following surgical castration.[11] Fisher and others[29] reported that plasma cortisol response was significantly reduced during the first 90 minutes following surgical or Burdizzo emasculatome castration in calves when the

lidocaine was administered prior to the procedure. Lidocaine is a commonly used local anesthetic that has a fast onset of action of 10-15 minutes, and an intermediate duration of action of 60–120 minutes.[30,31] Based on these data, increases in plasma cortisol are believed to reflect pain experienced as a result of castration.[4]

Cortisol has been widely used as a measurement of distress since its response magnitude, as indicated by peak height, response duration and/or integrated response usually accords with the predicted noxiousness of different procedures.[9] At each end of the cortisol response range, however, interpretation is less straightforward. At the lower end, for example, studies have shown that tail docking with a ring and tail docking with a docking iron cause similar cortisol responses to control handling in older lambs.[32] At the upper end of the range, there are several examples where cortisol responses do not increase proportionally to the severity of different treatments as might be expected. This may suggest a "ceiling effect" on plasma cortisol responses.[32,33] Other studies have shown that plasma cortisol concentrations following surgical castrations vary greatly between animals.[27] Based on these data, it has been hypothesized that low responses may be due to individuals having high pain thresholds.[11] Variations may also come about due to differences in the way in which a particular castration method is performed by different operators. These data suggest that plasma cortisol levels may not always accurately reflect the extent of the pain response in animals.

Substance P. Substance P (SP) is an 11-amino acid prototypic neuropeptide that regulates the excitability of dorsal horn nocireceptive neurons and is present in areas of the neuroaxis involved in the integration of pain, stress, and anxiety. Studies have shown that plasma SP levels are up to 27-fold greater in human patients with soft tissue injury than healthy controls.[34] Our research group recently conducted a study to evaluate plasma SP and cortisol response following castration.[35] Calves were acclimated for 5 days prior to random assignment based on scrotal circumference to a castration or uncastrated control group. Blood samples were collected at -24, -12, and 0 hours pre-castration and 3, 10, 20, 30, 45, 60, 90, 120, 150, 180, and 240 minutes post-castration. Vocalization and attitude scores were determined at the time of castration or simulated castration. Plasma SP and cortisol were determined using competitive and chemiluminescent enzyme immunoassay, respectively. Data were analyzed by repeated measures analysis using a mixed effects model allowing for unequal variances. No significant difference in plasma cortisol response between castrated and uncastrated calves was observed over time ($p=0.644$). In contrast, mean plasma SP concentrations were significantly higher in castrated calves compared to uncastrated controls over the course of the study ($p=0.042$). Cortisol responses over time in calves with vocalization scores of 0 were not significantly different from calves with vocalization scores of 3 ($p=0.17$). However, calves with vocalization scores of 3 had significantly higher SP levels when compared to calves with scores of 0 ($p=0.033$). These findings contradict previous reports that show an increase in plasma cortisol relative to pain post-castration. Significant increases in plasma SP concentration post-castration suggest that this measurement may be associated with nociception; however, further investigation is necessary.

Accelerometers. Accelerometers have been used in other species to detect lameness[36] and remotely monitor level of animal activity.[37] Our research group has utilized video observations to determine the accuracy of accelerometers to measure behavior changes in cattle and to determine differences in beef bull behavior post-castration.[38] Three Holstein calves and 12 healthy beef bulls had two-dimensional accelerometers placed on three animals and data were logged simultaneous to video recording of animal behavior. The subsequent data set was used to generate and validate a predictive model to classify animal posture (standing or lying) and type of activity (standing in place, walking, eating, getting up, lying awake, or lying sleeping). The algorithms developed were used to conduct a prospective trial to determine differences in bull behavior in the first 24 hours post-castration ($N=6$) compared to both control animals (non-castrated) ($N=6$) and pre-castration readings from the same bulls. Based on the analysis of the 2-D accelerometer signal, posture can be classified with a high degree of accuracy (98.3%) and the specific activity can be estimated with a reasonably low misclassification rate

(23.5%). Employing the system to compare behavior post-castration revealed that castrated calves spent a larger ($P < 0.05$) amount of time standing (79.3%) compared to either pre-castration readings (51.2%) or control calves after castration (64.3%). Animals also spent a lower percentage of the time eating in the post-castration phase. The 2-D accelerometers provided accurate classification of animal posture and reasonable classification of animal activity. Collected data allowed quantification of behavioral differences between animals after a surgical procedure and provides a valuable tool to compare research with behavioral endpoints.

Radar speed cameras. Burrows and Dillon[39] and Fell et al.[40] used radar speed cameras to measure the speed of cattle exiting a squeeze chute. Cattle with faster exit speeds had lower weight gains, more sickness, and more dark cutting meat. The major problem with chute exit speed as a means to determine pain is that it does not work well for highly acclimated, very tame animals such a dairy cattle. There are also certain breeds such as *Bos indicus* breeds that are more prone to demonstrate rapid chute exit speeds when compared with *Bos taurus* breeds.

Pain management in cattle

It has been suggested that a surgical stimulus such as castration in calves is so brief that little difference can be observed or measured between animals having or not having local anesthetic applied.[41] However, alleviating pain associated with surgical castration by administration of local anesthesia increased weight gain in cattle for 35 days following castration.[29] This suggests that alleviating acute pain at the time of castration may have economic benefit.[11] Ketoprofen, a NSAID analgesic not approved for use in cattle in the U.S., has been shown to reduce acute plasma cortisol response in cattle following administration at the time of castration.[26-28] Giving both a local anesthetic and intravenous ketoprofen before surgery-cut castration was found to virtually abolish the post-surgery cortisol response.[27,28] Ketoprofen given alone was also found to reduce the plasma cortisol response to Burdizzo emasculatome castration more effectively than a local anesthetic or an epidural.[28] Similar studies examining NSAIDs that are approved for use in food-producing animals in the U.S. have not been conducted. Furthermore, all these studies examining the efficacy of analgesic drugs in farm animals fail to report associated plasma drug concentrations essential for designing efficacious analgesic regimens. Some of the parameters described above may be useful to allow us to determine the efficacy of analgesics in food animals.

Our research group recently conducted a study to examine the effect of oral aspirin and intravenous sodium salicylate on acute plasma cortisol response following surgical castration.[33] Twenty bulls, randomly assigned to the following groups: 1) uncastrated, untreated controls, 2) castrated, untreated controls, 3) 50mg/kg sodium salicylate IV pre-castration and 4) 50mg/kg aspirin (acetylsalicylic acid) per os pre-castration, were blood sampled at 3, 10, 20, 30, 40, 50 minutes and 1, 1.5, 2, 4, 6, 8, 10 and 12 hours post-castration. Samples were analyzed by competitive chemiluminescent immunoassay and fluorescence polarization immunoassay for cortisol and salicylate respectively. Data were analyzed using noncompartmental analysis, a simple cosine model, ANOVA and t-tests. Intravenous salicylate Vd_{ss} was 0.18 L/kg, Cl_B was 3.36 mL/min/kg and $T_{½\lambda}$ was 0.63 hours. Plasma salicylate concentrations above 25 µg/mL coincided with significant attenuation in peak cortisol concentrations ($p=0.029$). Peak salicylate concentrations following oral aspirin administration was less than 10 µg/mL and failed to attenuate cortisol response. Once salicylate concentrations decreased below 5 µg/mL, cortisol response in the castrated groups were significantly higher than uncastrated controls ($p=0.018$). To our knowledge this is the first study relating plasma analgesic drug concentrations directly to mitigation of plasma cortisol response post-castration. These findings have important implications for designing effective analgesic regimens to alleviate the stress response associated with painful routine animal husbandry procedures.

A protocol for use of IM butorphanol/xylazine/ketamine (BXK) was presented by Dr. Matt Miesner, with credit given to Dr. Eric Abrahamsen, at the 2007 Kansas State University June Conference.[42] The regimen consists of butorphanol (0.01–0.025 mg/kg) + xylazine (0.02–0.05 mg/kg) + ketamine (0.04–0.1 mg/kg). Dr. Miesner noted that for a 450 kg animal, 5 mg butorphanol, 10 mg xylazine, and 20 mg ketamine would constitute the low end of the dosing range. **Note that the**

calculation should involve 2x xylazine as compared to butorphanol and 2x ketamine as compared with xylazine. They have noted up to an hour of cooperation using this protocol but more fractious patients may require increased doses. Dr. Miesner suggests giving no more than 10 mg butorphanol or 20 mg of xylazine the initial dose (this would be for the 450 kg animal on up).

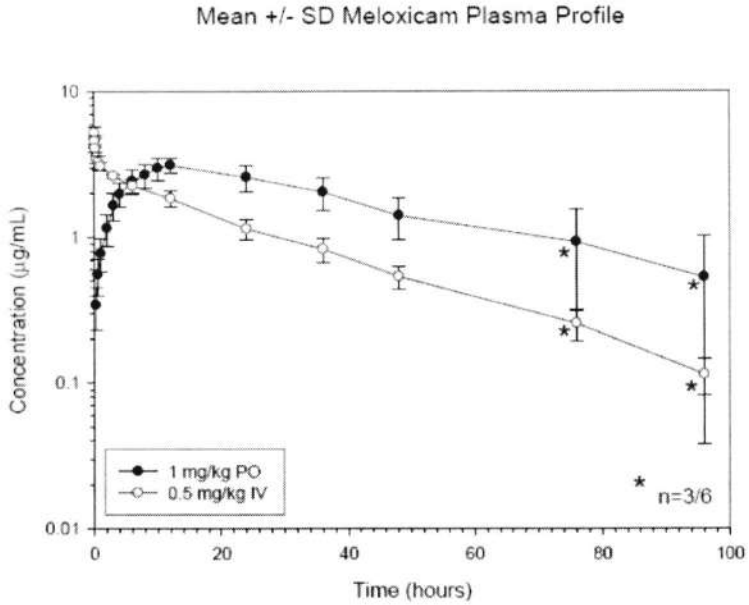
Stun "recipe" for large groups of cattle:

- 5 ml ketamine (100mg/mL solution)
- 10 ml **SMALL ANIMAL!** xylazine (20 mg/ mL)
- 10 ml of butorphanol (10 mg/mL)
- Makes a 25 mL stock solution to be used at one time due to stability concerns
- Docile (dairy) cattle- 1 mL/ 400 Kg (880 lbs)
- Fractious (beef) cattle- 1 mL/ 200 kg (440 lbs)
- Onset: IV faster than IM faster than SC
- Duration of effect: SC > IM > IV
- Risk of recumbency: IV> IM > SC (High dose > Low dose)

Meloxicam

Meloxicam is a NSAID of the oxicam class that is approved in the European Union for adjunctive therapy of acute respiratory disease; diarrhea and acute mastitis when administered at 0.5 mg/kg IV or SC. Meloxicam is considered to bind preferentially to cyclooxygenase-2 (COX-2) inhibiting prostaglandin synthesis although definitive evidence of COX-selectivity in calves is deficient in the published literature. Heinrich et al.[5] demonstrated that 0.5 mg/ kg meloxicam IM combined with a cornual nerve block reduced serum cortisol response for 6 hours in 6-12 wk old calves compared with calves receiving only local anesthesia prior to cautery dehorning. Furthermore, calves receiving meloxicam had lower heart rates and respiratory rates than placebo treated control calves over 24 hours post-dehorning. Stewart et al.[6] found that meloxicam administered IV at 0.5 mg/kg mitigated the onset of pain responses associated with hot-iron dehorning in 33 ± 3 day old calves compared with administration of a cornual nerve block alone as measured by heart rate variability and eye temperature. These findings indicate that administration of meloxicam at 0.5 mg/kg IV or IM decreases physiological responses that may be linked to pain and distress associated with cautery dehorning in preweaning calves.

The purpose of this study was to investigate the pharmacokinetics and oral bioavailability of meloxicam in ruminant calves.[7] Six Holstein calves (145–170 kg) received either meloxicam IV at 0.5 mg/kg or oral meloxicam at 1 mg/kg in a randomized cross-over design with a 10-day washout period. Plasma samples collected up to 96 hours post-administration were analyzed by LC-MS followed by noncompartmental pharmacokinetic analysis. A mean peak plasma concentration (Cmax) of 3.10 ug/mL (range: 2.64–3.79 ug/mL) was recorded at 11.64 hours (range: 10–12 hours) with a half-life (T ½ λz) of 27.54 hours (range: 19.97–43.29 hours) after oral meloxicam administration. The bioavailability (F) of oral meloxicam corrected for dose was 1.00 (range: 0.64–1.66). These findings indicate that oral meloxicam administration could be an effective and convenient means of providing long-lasting analgesia to ruminant calves.



In the U.S., meloxicam administered to cattle by any route constitutes extra-label drug use (ELDU). Under the Animal Medicinal Drug Use Clarification Act (AMDUCA), ELDU is permitted for relief of suffering in cattle provided specific conditions are met. These conditions include that (1) ELDU is permitted only by or under the supervision of a veterinarian, (2) ELDU is allowed only for FDA approved animal and human drugs; (3) ELDU is only permitted when the health of the animal is threatened and not production purposes; (4) ELDU in feed is prohibited and (5) ELDU is not permitted if it results in a violative food residue. Therefore, use of oral meloxicam to alleviate suffering associated with dehorning and castration in calves in the U.S. would be required by law to comply with these regulations.

Currently the only NSAID approved for use in cattle in the U.S. is flunixin meglumine. The plasma elimination half-life of flunixin is reported to be 3–8 hours therefore requiring once daily administration. Although this drug class is recognized as having analgesic properties, flunixin is only indicated for control of fever associated with respiratory disease or mastitis, and fever and inflammation associated with endotoxemia, rather than for control of pain. Studies demonstrating the analgesic effects of flunixin at the approved dose of 2.2 mg/kg are deficient in the published literature. Use of flunixin meglumine is further complicated by the requirement for intravenous administration which is more stressful on the animal and involves more skill and training on the part of the operator. Several reports have suggested that the IM administration of flunixin may result in significant myonecrosis and tissue residues. In the absence of data demonstrating that flunixin reduces signs of pain and distress associated with dehorning and castration in calves, it could be argued that use of oral meloxicam for this purpose can be justified under AMDUCA. Meloxicam (20 mg/ml) is approved for use in cattle in several European countries with a 15 day meat and five day milk withdrawal time following administration of 0.5 mg/kg IM or SC. An oral meloxicam suspension (1.5 mg/mL) and injectable formulation (5 mg/mL) are approved in the U.S. for the control of pain and inflammation associated with osteoarthritis in dogs. Furthermore, an injectable formulation (5 mg/ml) is approved for the control of post-operative pain and inflammation in cats. Several generic tablet formulations containing meloxicam (7.5 and 15 mg) have recently been approved for relief of signs and symptoms of osteoarthritis in human medicine. The cost of administering IV meloxicam to calves in the present study was approximately U.S. $58.00/100 kg bodyweight and the cost of administering oral meloxicam was U.S. $0.30/100 kg bodyweight.

Gabapentin

Gabapentin is a γ-aminobutyric acid (GABA) analogue indicated for treatment of neuropathic pain. This study determined the pharmacokinetics of oral gabapentin alone or in combination with meloxicam in ruminant calves. Gabapentin capsules at 10 mg/kg PO or gabapentin powder (from capsules) and meloxicam tablets at 15 mg/kg and 0.5 mg/kg PO, respectively was administered to six beef calves. Plasma drug concentrations were determined over 48 h post-administration by liquid chromatography/mass spectrometry followed by non-compartmental pharmacokinetic analysis. The mean (±SD) *C*max, *T*max and elimination half-life ($t_{½}$ λz) for gabapentin (10 mg/kg) alone was 2.97±0.40 μg/mL, 9.33±2.73 h and 11.02±3.68 h, respectively. The mean (±SD) *C*max, *t*max and $t_{½}$ λz for gabapentin (15 mg/kg) co-administered with meloxicam was 3.57±1.04 μg/mL, 7.33±1.63 h and 8.12±2.11 h, respectively. The mean (±SD) *C*max, *T*max and $t_{½}$ λz for meloxicam was 2.11 ± 0.19 μg/mL, 11.67 ± 3.44 h and 20.47 ± 9.22 h, respectively. Plasma gabapentin concentrations >2 μg/mL were maintained for up to 15 h and meloxicam concentrations >0.2 μg/mL for up to 48 h. The pharmacokinetic profile of oral gabapentin and meloxicam supports clinical evaluation of these compounds for management of neuropathic pain in cattle.

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Bull biosecurity: Diagnosing pathogens that cause infertility of bulls or transmission via semen

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Summary

Appropriate biosecurity measures to prevent bulls or cryopreserved semen from causing infertility or transmitting pathogens equates to performing an acceptable diagnostic test on an appropriate sample at an appropriate time to ensure the absence of particular pathogens. The goal of this review is to concisely describe appropriate biosecurity measures for bulls and semen that originate in the United States. Appropriate biosecurity measures to prevent the transmission of *Brucella abortus*, *Campylobacter fetus*, *Histophilus somni*, *Leptospira* species, *Mycobacterium bovis*, *Mycobacterium avium* subsp. *paratuberculosis*, *Tritrichomonas foetus*, bovine viral diarrhea virus, bluetongue virus, infectious bovine rhinotracheitis virus (bovine herpesvirus-1), and bovine herpesvirus-5 are described. While some determinants may cause producers to neglect desirable biosecurity measures when introducing bulls or semen to breeding herds, informed practitioners can often minimize disease risks by facilitating selection of sires or semen from low risk sources.

Keywords: Pathogen, diagnostics, venereal, infertility

Introduction

Introduction of novel genetics to improve the performance of cattle herds is most commonly and efficiently achieved through introduction of new sires. Unfortunately, the introduction of new sires via natural breeding or artificial insemination has the potential to introduce pathogens that may result in infertility or subfertility and transmit disease causing agents to naive cows or heifers that may exhibit additional morbidity or mortality. Pathogens that should receive consideration when introducing new bulls in the United States include *Brucella abortus*, *Campylobacter fetus*, *Histophilus somni*, *Leptospira* species, *Mycobacterium bovis*, *Mycobacterium avium* subsp. *paratuberculosis*, *Tritrichomonas foetus*, bovine viral diarrhea virus, bluetongue virus, infectious bovine rhinotracheitis virus (bovine herpesvirus-1), and bovine herpesvirus-5. When obtaining bulls or semen from outside of the United States, biosecurity measures to prevent introduction of foot-and-mouth disease, rinderpest, lumpy skin disease, and Rift Valley fever viruses should also be considered.[1] Prudent use of resources may dictate that new bulls are only tested for some of these pathogens due to (a) low prevalence or total lack of the pathogen in the herd from which the bull originated, (b) a high likelihood that the pathogen is already present in the herd to which the bull will be introduced, (c) the lack of diagnostic sensitivity of available assays to detect particular pathogens in bulls, and (d) the time between when the owner first has access to diagnostic samples from the bull and when the bull needs to be introduced to optimize reproduction of the herd.

Brucella abortus

Localization of *Brucella abortus* in the reproductive tract of the bull can result in production of semen containing the bacteria. While the bull appears to play a minor role in the spread of *Brucella*, semen from infected bulls has been demonstrated to result in transmission to susceptible cows.[1] The epididymis, seminal vesicle, and testicle (to a lesser degree) contain significant concentrations of erythritol, a polyhydric alcohol which enhances the growth of *B. abortus* resulting in localized inflammation and infertility of bulls. Bulls to be used for natural breeding should be obtained from herds or states that are certified as brucella-free and should not exhibit orchitis or epididymitis. To achieve greater biosecurity and fulfill the minimum requirements of Certified Semen Services (CSS), a serologic test to detect brucellosis—specifically a buffered antigen plate agglutination (BPAT) test, card test or complement fixation test—shall be negative within 30 days prior of entry into the isolation facility and at least 30 days after the pre-isolation test. While all three of these diagnostic tests exhibit acceptable sensitivities to detect brucellosis, the BPAT yields the highest sensitivity and highest specificity.[2]

Campylobacter fetus

Infections of bulls with *Campylobacter fetus* subsp. *venerealis* are asymptomatic. Infections of heifers and cows cause infertility and early embryonic death. Transmission usually occurs via natural mating or artificial insemination using contaminated semen lacking antibiotics in extender.[3] As vaccination of bulls up to five years of age using a bacterin in an oil emulsion adjuvant is considered both protective and curative, vaccination prior to natural mating may be considered the least expensive method of biosecurity against *C. fetus*. Minimum requirements for production of semen under CSS guidelines dictate that washings or scrapings of preputial smega from bulls shall be negative for *C. fetus* using fluorescent antibody screening tests or cultures. To achieve this optimal biosecurity, the minimum number of negative tests at weekly intervals varies with up to six test negative results necessary to validate the specific pathogen free status of bulls over one year of age. Recently, PCR-based assays have been developed as rapid screening tests for the detection of *C. fetus*. Research involving a quantitative PCR assay for the 5′ *Taq* nuclease of *C. fetus* subsp. *venerealis* indicates that PCR may be more analytically and diagnostically sensitive that culture.[4] Notably, whether using a PCR assay or culture, collection of preputial samples using a 7.5-cm long, 8-mm diameter polyethylene corrugated scraper head ("bull rasper") increased the diagnostic sensitivity of the detection method by facilitating collection of more bacteria from infected bulls.[4]

Histophilus somni

The association of *Histophilus somni* (previously *Hemophilus somnus*) with bovine abortion and infertility is controversial and may depend on the production of specific virulence factors by the bacterial strain. This fastidious gram-negative rod was isolated from the prepuce, bladder, or accessory sex glands of 24 of 31 (77%) bulls from an Ontario slaughterhouse.[5] Carrier bulls can infect cows as this commensal organism or opportunistic pathogen—depending on the host, environment, and one's interpretation of prior research regarding this organism—readily spreads via natural breeding. Adding antibiotics to extended semen or treating bulls with appropriate antibiotics may control the spread of this potential pathogen. While administration of oxytetracycline to bulls has been implemented by some herds to prevent introduction of infected bulls, antibiotic sensitivity testing indicates some resistence of *H. somni* to oxytetracycline. Interpretation of serologic antibody titers to *H. somni* is difficult and may require assessment of paired serum samples. Titers between 1:256 and 1:512 in nonvaccinated cows have been attributed to early active or chronic infections while titers between 1:1040 and 1:4096 have been attributed to active infections.

***Leptospira* species**

While pathogenic *Leptosprira* species are mainly shed in urine, transmission of spirochetes is possible via semen.[6] Similar to controlling *H. somni*, adding antibiotics to extended semen or rationally treating infected bulls with appropriate antibiotics may control the spread of this pathogen.[1] If leptospira vaccines have not been previously administered to bulls, serologic testing may indicate lack of exposure to the five most common pathogenic serotypes. Minimum requirements for production of semen under CSS guidelines dictate that bulls are seronegative within 30 days prior to entry or at least exhibit a stabilized low titer ($\leq$ 1:400) on two tests at least two to four weeks apart before entering the isolation facility.

Mycobacterium bovis

Bovine tuberculosis caused by *Mycobacterium bovis* and rarely *M. tuberculosis* provides a significant risk for human infection. Transmission of this pathogen via semen is possible.[6] Bulls to be used for natural breeding should be obtained from herds or states that are certified as bovine tuberculosis-free. To achieve greater biosecurity as is required by CSS guidelines, an intradermal tuberculin test shall be negative within 60 days prior to entry into the isolation facility and at least 60 days after the pre-isolation test.

Mycobacterium avium subsp. *paratuberculosis*

Paratuberculosis or Johne's disease is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Although the organism has been isolated from testicular tissue and semen of infected bulls, venereal transmission has been considered to be of negligible importance epidemiologically. Despite some investigation, transmission by contaminated semen or semen from contaminated bulls has never been demonstrated.[6] Semen is considered to contain a low concentration of infectious MAP as only eight of 31 semen samples from a bull exhibiting clinical signs and one of 100 semen samples from a subclinically infected bull yielded MAP in culture.[7,8] In contrast, semen collected over three years from a subclinically infected bull intermittently yielded high concentrations of MAP DNA which caused the authors to speculate that semen might be epidemiologically significant if the pathogen had entered the previously described state of being viable but non-cultivable.[9] To achieve sufficient biosecurity for MAP in bulls that will be introduced into herds for natural breeding, a valid history of absence of paratuberculosis in the herd from which the bull originates is considered to be of equal or greater importance than negative results using currently available diagnostic tests on individual bulls less than two years of age.

Tritrichomonas foetus

Similar to infections with *Campylobacter fetus* subsp. *venerealis*, infections of bulls with *Tritrichomonas foetus* are asymptomatic. Infections of heifers and cows with *T. foetus* cause transient infertility, early embryonic death, abortion, and pyometra. Transmission usually occurs via contact associated with natural mating; however, the protozoan may be present in semen and transmission via semen has been demonstrated.[1] Practitioners and producers should clearly understand that the required regulatory testing for the sale of mature bulls in many states serves as a deterrent to selling known positive bulls but is not stringent enough to ensure a high degree of biosecurity. Minimum requirements for production of semen under CSS guidelines dictate that washings or scrapings of preputial smega from bulls shall be negative for *T. foetus* using microscopic examinations of cultured preputional material collected from the preputial fornix. To achieve this optimal biosecurity, the minimum number of negative tests at weekly intervals varies with up to six test negative results necessary to validate the *T. foetus*-free status of bulls over one year of age. Recent research demonstrated that a gel-based PCR and microscopic examinations of cultured preputional material were functionally equivalent methods to detect *T. foetus* if storage and transport temperatures can be appropriately controlled.[10,11] Results suggested that when using cultured specimens for *T. foetus* diagnostic purposes, a combination of culture and a gel-based PCR assay performed on three sequential preputial scrapings was the best method for identifying infected bulls during a naturally occurring herd outbreak.[11] Trichomonads other than *T. foetus* can be present in a preputial sample and may result in false positives when relying only on microscopic examination of cultured preputial samples.[12] A staining technique or PCR assay can be useful in differentiating *T. foetus* from other trichomonads observed in samples from virgin bulls.

Bovine viral diarrhea virus

Infections of cattle with bovine viral diarrhea virus (BVDV) can cause disease which ranges from subclinical to severe. From semen collected from bulls exhibiting a persistent infection, an acute infection, or a persistent testicular infection, the virus has been isolated and can result in transmission.[13-15] At a minimum, biosecurity measures may include testing bulls for persistent infection via a validated PCR assay of ear notch tissues, serum or whole blood; a validated antigen capture ELISA assay of ear notch tissues, whole blood, or serum; or validated immunohistochemical staining of ear notch tissues.[16-18] To prevent acute infections from spreading BVDV via semen, bulls should be isolated from contact with novel cattle for at least 21 days before entering the breeding herd. To prevent rare persistent testicular infections from transmitting BVDV to naïve heifers and cows, semen should be assayed for BVDV using validated PCR or virus isolation tests of semen. The CSS minimum requirements for preventing contamination of processed and cryopreserved semen with BVDV are summarized in Figure 1.

Figure 1. Certified Semen Services (CSS) minimum requirements for preventing bovine viral diarrhea virus contamination of semen produced for artificial insemination.

Preventing persistent infections from contaminating semen	Preventing acute infections from contaminating semen	Preventing persistent testicular infections from contaminating semen
1 Within 30 d prior to isolation entry, ☑ negative by VI, PCR, or ACE of whole blood or serum (if ≥ 6 months of age).	**2** No sooner than 10 d after isolation entry, negative by ☑ PCR on whole blood, or VI with one pass coupled with FA or IP on whole blood or serum (if ≥ 6 months of age).	**7** Before release of semen, test bulls by SN for antibodies to both types I and II. ☑ If seropositive, must have negative test on processed semen by PCR or VI with one pass coupled with FA or IP viral detection.
2 No sooner than 10 d after isolation entry, negative by ☑ PCR on whole blood, or VI with one pass coupled with FA or IP on whole blood or serum (if ≥ 6 months of age).	**3** Only if # 2 is positive, then animal is isolated from all other cattle and retested ☑ in not less than 21 d by PCR on serum or VI with one pass coupled with FA or IP on whole blood or serum (if ≥ 6 months of age).	**Alt.** Before release of semen, **7** all bulls must have negative test on processed semen by PCR or VI with ☑ one pass coupled with FA or IP viral detection.
3 Only if # 2 is positive, then animal is isolated from all other cattle and retested ☑ in not less than 21 d by PCR on serum or VI with one pass coupled with FA or IP on whole blood or serum (if ≥ 6 months of age).	**4** If #3 is positive, then the animal is not eligible to enter the ☒ resident herd of the CSS-approved AI center.	**8** If processed semen in 7 or Alt. 7 tests positive by VI, then additional ☑ processed semen should be tested to confirm persistent testicular infection.
4 If #3 is positive, then the animal is not eligible to enter the ☒ resident herd of the CSS-approved AI center.	**5** If #3 is negative, then animal remains isolated from all other cattle until it achieves ☑ 2 negative tests at least 10 d apart by VI with one pass coupled with FA or IP on whole blood or serum (if ≥ 6 months of age).	**9** Any bull exhibiting a persistent testicular infection for BVDV is not ☒ eligible for semen collection and is not permitted to remain in the resident herd.
	6 If #5 is achieved, then any semen collected and processed ☑ within 30 days before or after the positive VI must be test negative by VI or PCR before distribution.	

VI = virus isolation; PCR = polymerase chain reaction; ACE = antigen capture ELISA (enzyme linked immunosorbent assay); FA = fluorescent antibody; IP = immunoperoxidase; AI = artificial insemination; SN = serum neutralization; Alt. = Alternate.

Bluetongue virus

Many infections of cattle with bluetongue fail to produce clinical signs of disease; however, viral infections may cause fever, facial edema, hemorrhages and ulceration of the mucous membranes. Some of the 26 serotypes of bluetongue virus such as BTV-8 are associated with more severe clinical signs. Bluetongue virus can be detected in the semen of viremic bulls and may result in viral transmission.[1,6] Some researchers hypothesize that transmission via cryopreserved semen may have initiated the BTV-8 epizootic in north-western Europe in 2006.[19] Therefore, the detection of circulating anti-BTV antibodies in bulls in semen collection centers may impede or prevent international trade of semen to countries free of BTV. To facilitate trade, the European Commission Regulation 1266/2007/EC states that semen may be imported if, for at least 60 days before and during collection of semen, (a) bulls are kept outside of an endemic zone, (b) bulls are protected against biting midges which spread the virus, or (c) bulls are kept during the seasonally midge-free period in a bluetongue seasonally-free area.[20] Semen may also be imported from bulls which test seronegative every 60 days or test free of bluetongue by PCR every 28 days according to the World Animal Health Organization (OIE) Terrestrial Animal Health Code.[20]

Bovine herpes virus-1

Bovine herpes virus-1 causes economically significant respiratory and reproductive loss in cattle. This alphaherpesvirus can be detected in semen and can result in viral transmission. Insemination of naïve heifers and cows with semen containing BHV-1 can cause endometritis, shortened inter-estral intervals and reduced conception rates.[21] Beyond the initial phase of infection, BHV-1 remains latent in sacral ganglia and a protracted course of intermittent virus excretion in seminal plasma may follow.[22] As a general rule, the site of primary infection generally determines the site of latency in local sensory ganglia; thus, one would expect that BHV-1 from an intranasal infection usually would not lie latent in sacral ganglia.[22] The intermittent shedding of BHV-1 in semen due to latent infections has caused some European countries to require that all bulls producing semen for import and all bulls in their domestic artificial centers must be seronegative for BHV-1.[1] For bulls to be used for natural breeding purposes in the United States, quarantine of bulls for at least 21 days limits the risk of BHV-1 shedding to contacted cows and heifers due to an acute infection in the bull. Vaccination of bulls to be used for natural breeding in the United States is recommended at least 28 days prior to introduction into the breeding herd. To optimize trade opportunities, bulls from which semen may be shipped internationally are ideally maintained as seronegative and not vaccinated for BHV-1.

Bovine herpes virus-5

Bovine Herpes Virus-5 (previously BHV-1.3) shares antigenic similarity to BHV-1 but has been associated with a fatal meningoencephalitis in calves. This virus has been detected in semen using PCR and virus isolation techniques.[23,24] Poor conception rates and pustular vulvovaginitis has been described in isolated cows and heifers artificially inseminated with semen containing BHV-5.[23] Although genomic and pathogenic differences between BHV-1 and BHV-5 are quite consistent, the two related viruses display extensive serological cross-reactivity which can be evidenced in serum neutralization tests.[25] Therefore, control measures to prevent contamination of semen with BHV-5 are the same as preventive measures for BHV-1.

Conclusions

The implementation of appropriate biosecurity measures for pathogens that exhibit the potential to cause infertility in cattle or may be transmitted via semen will consistently prevent the spread of animal disease. Bulls to be used in the United States should be prudently quarantined and tested appropriately for the described pathogens prior to introduction for natural breeding. Using semen collected under CSS minimum requirements for production provides assurance that appropriate biosecurity procedures have been applied. Appropriate biosecurity measures constitute using the least cumbersome management tools and diagnostic tests that will facilitate optimal trade in pathogen-free bulls and semen.

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Assessment of available vaccines for bulls to prevent transmission of reproductive pathogens

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Summary

Immunization of bulls to prevent infertility or transmission of reproductive pathogens equates to the prudent use of available vaccines to achieve effective immunity prior to exposures that will infect reproductive tissues or negatively impact reproductive organs. The goal of this review is to concisely describe appropriate immunization for bulls used for natural breeding and semen production in the United States. Appropriate vaccination practices to prevent the transmission of *Campylobacter fetus*, *Histophilus somni*, *Leptospira* species, *Tritrichomonas foetus*, bovine viral diarrhea virus, infectious bovine rhinotracheitis virus (bovine herpesvirus-1), and bovine herpesvirus-5 are described. In the United States, licensed vaccines are not available or vaccination is usually contraindicated for *Brucella abortus*, *Mycobacterium bovis*, *Mycobacterium avium* subsp. *paratuberculosis*, and bluetongue virus. While some determinants may cause producers to neglect immunization of bulls for reproductive pathogens, informed practitioners can often minimize disease risks by recommending timely vaccination of future sires with appropriate vaccines.

Keywords: Pathogen, vaccination, immunization, venereal, infertility

Introduction

Effective and timely immunization of sires to be used for natural breeding or artificial insemination has the potential to prevent infertility or subfertility and transmission of some reproductive pathogens. Pathogens that should receive consideration when introducing new bulls in the United States include *Brucella abortus*, *Campylobacter fetus*, *Histophilus somni*, *Leptospira* species, *Mycobacterium bovis*, *Mycobacterium avium* subsp. *paratuberculosis*, *Tritrichomonas foetus*, bovine viral diarrhea virus (BVDV), bluetongue virus, infectious bovine rhinotracheitis virus (bovine herpesvirus-1; BHV-1), and bovine herpesvirus-5 (BHV-5). Unfortunately, vaccines for which efficacy and safety have been proven are only available to prevent the negative consequences associated with some of these disease causing agents. Effective vaccination strategies may be limited by the safety of available vaccines, the inability to administer vaccines at the appropriate time, and the lack of demonstrated efficacy to prevent reproductive sequelae.

Brucella abortus

Localization of field strains of *Brucella abortus* in the reproductive tract of the bull can result in orchitis, testicular degeneration and production of semen containing the bacteria. Vaccination of heifers between four and ten months of age is performed in the United States to mitigate perceived disease risks associated with *B. abortus*, minimize regulatory issues related to interstate transportation of breeding age animals, or increase market value of heifers. Bulls to be used for natural breeding should not be vaccinated unless the risk of exposure to *B. abortus* is very high. Intramuscular vaccination of six sexually mature bulls with strain RB51 did not result in shedding of the organism, prolonged colonization, or reproductive problems based on serial breeding soundness examinations during the five weeks after vaccination.[1] As strain RB51 is essentially devoid of the O-polysaccharide chain, animals vaccinated with RB51 do not produce antibodies directed against the O-polysaccharide chain and can be distinguished from animals infected with field strains or vaccinated with strain 19. Clearly bulls should not be administered vaccines containing strain 19 as vaccination may cause orchitis, enduring testicular infections, and a seropositive status that cannot be differentiated from exposed bulls.[1,2] Vaccination of bulls to be maintained in bull studs is rarely if ever indicated.

Campylobacter fetus

Infections of bulls with *Campylobacter fetus* subsp. *venerealis* are asymptomatic while infections of heifers and cows cause infertility and early embryonic death. As vaccination of bulls up to five years of age using a bacterin in an oil emulsion adjuvant is considered both protective and curative, vaccination prior to natural mating may be considered the least expensive method of biosecurity against *C. fetus*. Unfortunately, vaccination may not result in every bull clearing the organism and some bulls may not clear the organism until more than six weeks after primary vaccination.[3,4] While vaccines containing oil emulsion adjuvants may cause notable tissue reactions, *C. fetus* vaccines containing an aluminum hydroxide adjuvant have not demonstrated acceptable efficacy in a field trial involving heifers.[5] Common recommendations for bulls that are considered to be at risk for exposure to and spreading of *C. fetus* subsp. *venerealis* involve an initial two-dose series of vaccinations (two to four weeks apart and at least 30 days before initiation of the breeding season) followed by annual vaccination.

Histophilus somni

The association of *Histophilus somni* (previously *Hemophilus somnus*) with endometritis, infertility, and abortion in cattle is controversial and may depend on the production of specific virulence factors by the bacterial strain. While field investigations attribute reproductive disease to introduction of bulls harboring this gram-negative cocco-bacillus,[6] the efficacy of vaccination in preventing reproductive disease remains to be assessed and described. Unfortunately, the published body of evidence does not provide a consistent estimate of the direction and magnitude of effectiveness of the more common use of immunization to prevent respiratory disease due to *H. somni*.[7]

***Leptospira* species**

While pathogenic *Leptosprira* species are mainly shed in urine, transmission of this opportunistic reproductive pathogen is possible via semen.[8] While the serovar *pomona* is clearly associated with outbreaks of bovine abortion in North America, the association of North American *hardjo* types with bovine infertility has been diagnosed but is controversial.[9-11] Vaccination strategies to prevent reproductive losses caused by *Leptospira* commonly focus on immunization of heifers and cows. While a controlled field trial in the United Kingdom demonstrated the ability of a *hardjo* vaccine to prevent infertility in vaccinated cows within naturally infected herds,[12] a similar, large, randomized controlled field trial in the United States did not detect differences in pregnancy and calving rates between cows vaccinated with a hardjobovis vaccine and controls.[10] Generalities regarding vaccine efficacy are to be avoided as research involving one 5-way pentavalent vaccine demonstrated lack of efficacy[13] while research involving another 5-way pentavalent vaccine containing the same serovars and types of *Leptospira* demonstrated consistent protection against the same challenge strain and route of challenge.[14] Depending on the potential for exposure of specific herds and regional considerations regarding association of *Leptospira* infection with reduced reproductive health, producers may choose to vaccinate bulls with a polyvalent product with the first dose administered after six months of age. In these situations, bulls often receive an initial two-dose series followed by annual revaccination. Bulls to be admitted or maintained in bull studs should not be vaccinated for *Leptospira* species as serologic status is used as the primary indicator of a possible ongoing infection.

Mycobacterium bovis

Transmission of *Mycobacterium bovis* via semen is possible.[8] While much work focuses on the development and validation of safe and effective vaccines for *M. bovis*, a vaccine currently is not available for administration to cattle in the United States.[15]

Mycobacterium avium* subsp. *paratuberculosis

While *Mycobacterium avium* subsp. *paratuberculosis* (MAP) has been isolated from testicular tissue and semen of infected bulls, venereal transmission has been considered to be of negligible importance.[8] Vaccination of cattle in the United States for MAP is not common. One vaccine, Mycopar® (Boehringer Ingelheim, Fort Dodge, IA), is limited to use in specific herds approved by state animal

health officials. This approval varies among states and requires validation that MAP causes infections within the herd, negative tuberculin tests on all test-eligible animals, and a signed agreement by the herd owner and state animal health agency. With this approval, only replacement heifers and bull calves between one and 35 days of age are eligible to receive MAP vaccine. A protective effect of vaccination on MAP infection and clinical disease has been described.[16] Except for extra-ordinary situations, vaccination of bulls is to be avoided as it increases the likelihood of testing positive for bovine tuberculosis via the caudal-fold skin test and Johne's disease via antibody-based tests such as ELISA.[16]

Tritrichomonas foetus

Infections of bulls with *Tritrichomonas foetus* are asymptomatic, while infections of heifers and cows with *T. foetus* cause transient infertility, early embryonic death, abortion, and pyometra. Vaccination strategies to prevent reproductive losses caused by *T. foetus* commonly focus on immunization of heifers and cows. One vaccine, Trichguard® (Boehringer Ingelheim) which contains whole cell antigens, is licensed in the United States as an aid in the prevention of disease caused by *T. foetus*. In a randomized, controlled field trial, administration of two doses (three weeks apart) to heifers four weeks prior to a 45-day breeding season with infected bulls resulted in reduced embryonic and fetal losses and a shorter duration of infection.[17] Research regarding immunization of bulls with various vaccines to prevent or cure infections with *T. foetus* has produced variable results.[18-21] Systemic immunization with whole cell antigens has prevented colonization of the preputial and penile mucosa after experimental inoculation.[18,21] Epidemiologic studies are not available to assess the reproductive impact of using immunized bulls in infected herds. If bulls are to be used for natural breeding in potentially infected herds, vaccination of bulls as well as heifers and cows can limit the economic impact of exposure to this pathogen. If vaccination is to be performed, two doses (two to four weeks apart) are recommended with the second dose administered four weeks prior to the breeding season.

Bovine viral diarrhea virus

Infections of cattle with BVDV can cause disease which ranges from subclinical to severe. To avoid contamination of semen with BVDV, bulls should be protected from persistent infection, a poorly timed acute infection, prolonged testicular infection, and persistent testicular infection.[22] While appropriate immunization of the heifer or cow that will gestate the male fetus can prevent persistent infection of the resulting bull, vaccination of the bull cannot prevent persistent infection as this enduring infection of all tissues is initiated before approximately 125 days of gestation.[22,23]

Immunization can prevent contamination of semen due to an uncontrolled acute infection initiated shortly before natural breeding or semen collection.[24,25] Acutely infected bulls can shed BVDV in semen from two to 20 days after initiation of an acute infection.[26] Two studies demonstrate that vaccination of bulls with a single dose of vaccine (Bovi-shield Gold, Pfizer Animal Health, Kalamazoo, MI or Express FP, Boehringer Ingelheim) containing cytopathic, modified-live strains of BVDV can prevent contamination of semen due to acute infections.[24,25]

Acute infection of peri-pubertal and post-pubertal bulls with noncytopathic strains of BVDV can result in prolonged testicular infections.[27] Research demonstrates that prolonged testicular infections can result in detection of viral RNA in semen for 2.75 years with infectious virus replicating in testicular tissue for 12.5 months after initiation of acute infections.[27] While infectious virus can be detected in testicular tissue and viral RNA can be detected in semen for extremely long periods of time, the potential for viral transmission due to prolonged testicular infections appears to be limited.[27] Two studies demonstrate that vaccination of bulls with a single dose of vaccine (Bovi-shield Gold, Pfizer Animal Health or Express® FP, Boehringer Ingelheim) containing cytopathic, modified-live strains of BVDV can prevent prolonged testicular infections.[24,25]

The characteristics of exposure to a noncytopathic field strain of BVDV that result in persistent testicular infections remain to be fully understood. Until our understanding advances, the potential for prevention of persistent testicular infections via vaccination is unknown. The initiating exposure for persistent testicular infections precedes seven months of age and results in seropositive bulls.[28,29]

Persistent testicular infections result in contamination of semen with infectious BVDV for at least 11 months.[28,29] The infectious virus excreted in contaminated semen readily transmits infection to artificially inseminated seronegative heifers or cows.[30]

Some caution is necessary to avoid undesirable consequences of vaccinating bulls for BVDV. Appropriately timed immunization of naïve bulls with a vaccine containing cytopathic, modified-live strains of BVDV is to be recommended. The initial dose of vaccine should be administered at least 28 days before introduction to the breeding herd to allow time for complete development of protective immunity and prevent the contamination of semen with BVDV due to the short-term, transient shed of modified-live BVDV in semen which occurs for up to 10 days after vaccination.[24] Naïve bulls should not be administered the currently available modified-live vaccine containing noncytopathic BVDV as this vaccine has been demonstrated to cause prolonged testicular infections under field conditions.[31]

Bluetongue virus

Infections with bluetongue virus may sometimes cause fever, facial edema, hemorrhages and ulceration of the mucous membranes and transmission via semen.[8,32] Immunity to bluetongue is serotype-specific. While a recent European outbreak of bluetongue virus serotype 8 was rapidly controlled through voluntary and mandatory use of inactivated vaccines, vaccination in the United States should be avoided as immunization will result in a seropositive status for many years that will impede trans-boundary trade of semen.[33] Modified-live vaccines developed for sheep should not be used in cattle because of the risk of transmitting vaccine strains via insect vectors.

Bovine herpes virus-1

Bovine herpes virus-1 causes economically significant respiratory and reproductive loss in cattle and can be transmitted via semen. Vaccination is commonly avoided in bulls to be admitted to bull studs as the resulting seropositive status may impede trans-boundary trade of semen. Vaccination of bulls to be used for natural breeding in the United States is considered prudent in many herds. Vaccines should be administered at least 28 days prior to introduction into the breeding herd.

Bovine herpes virus-5

Bovine herpes virus-5 (previously BHV-1.3) has been detected in semen of infected bulls using PCR and virus isolation techniques.[36,37] Although genomic and pathogenic differences between BHV-1 and BHV-5 are quite consistent, the two related viruses display extensive serological cross-reactivity which can be evidenced in serum neutralization tests.[38] Therefore, immunization measures to prevent contamination of semen with BHV-5 are the same as measures for BHV-1.

Conclusions

The implementation of appropriate immunization programs for reproductive pathogens can aid in the prevention of disease transmission. Bulls to be used for natural breeding in the United States should be prudently vaccinated for some reproductive pathogens. A combination of appropriate biosecurity measures and limited use of vaccines constitutes the basis for disease control programs for bulls entering or maintained in bull studs.

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New AI industry paradigms for sire management and semen quality evaluation

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Abstract

The major paradigm shift faced by the bovine artificial insemination (AI) was the replacement of locally produced non-frozen liquid semen with cryopreserved semen collected and processed by any of hundreds of AI centers that existed at the time (late 50's, early 60's). The genetic progress realized by the dairy producer as result of this technology introduction has been astounding. However, improved efficiency and greater competition has led to consolidation among both AI centers and producers alike. The turn of the 21st century finds numerous new technologies poised to alter future paradigms of the AI industry. Albeit with less than desirable levels of efficiency, sex-sorted semen has become a commercial reality. Estrous detection has largely been replaced by tail-paint, ovulation synchronization, or computerized activity monitors. Automated semen evaluation technologies greatly enhance the precision of semen quality estimates and may enhance our understanding of previously unmeasurable attributes of semen quality. However, it is genomic selection that is most profoundly reshaping the new paradigms of AI and dairy industries. Today, the reliability of genomic predictions for milk production among newborn Holstein calves is equivalent to that obtained by 33 daughters in production under the traditional progeny test system. The accuracy of these estimates will continue grow as more animals are tested, which likely further support the trend of driving a greater percent of semen sales toward young sires. Stall space and efficiency of semen production will become of greater concern as the traditional proven sires are replaced by genomic young sires with ~30% the semen production capacity of their counterparts. Of paramount concern will be maintaining the phenotypic data necessary to continually ensure calibration of genomic predictions as producers seek to eliminate unnecessary operating expenses.

Keywords: Artificial insemination, sex-sorted semen, artificial insemination submission, genomic selection, semen quality, sire fertility

Introduction

The foundation of the AI industry was development of Dairy Herd Improvement Associations (DHIA) around the turn of the century. Although AI had previously been performed in several species for more than a century prior, DHIA provided the first organized approach to truly identify superior genetics and thereby provided the incentive for mass propagation. In the 1930's and 40's commercial bovine AI organizations were founded in Europe and across North America. The limited shelf life of non-frozen liquid semen dictated the development of local AI cooperatives, which numbered near 100 by the time of serendipitous discovery by Chris Polge that glycerol facilitates sperm cryosurvival. This discovery and the conversion from fresh to cryopreserved semen initiated the first major paradigm shift in the AI industry. Producers were no longer dependent on their local cooperative and could now purchase semen from any AI organization in the world. The resultant competition among AI organization ultimately resulted in a consolidation among AI organizations that has only recently begun to stabilize with about six major suppliers in North America and no more than couple dozen major suppliers world-wide. Although numerous other changes in dairy management have been adopted over the years, improved genetics has no doubt played a pivotal role in enhanced production efficiency that presently allow the US dairy industry to produce approximately 50% more total pounds of milk than in the 1940's and does so with only ~38% of the total cow population of the 1940's.

The turn of the 21st century finds the AI industry welcoming numerous new paradigms. Sex pre-selection of offspring has become a commercial reality. Numerous automated technologies have been adopted by AI centers to assist in the daunting task of large-scale commercial semen quality control. National and regional estimates of sire fertility have gained in popularity as a trait of primary emphasis. Furthermore and without question, genomic selection is revolutionizing the AI industry and will

undoubtedly result in the most far reaching new paradigm since the introduction of cryopreserved semen. The objective of this manuscript is to review the current status (symptoms?) of this paradigm of change in the bovine AI industry and provide some prognostications to the future.

Sex-sorted semen

The use of flow cytometery as a reliable and repeatable method to distinguish X- and Y-chromosome-bearing sperm was first reported provided by Garner and coworkers in 1983.[1] Johnson et al.[2] were the first to validate the technology with the birth of live offspring of the predicted sex. It was clear from the onset that this technology had the potential to consistently skew in the sex ratio of offspring toward 90% of the desired sex. However it was also equally evident that the highly invasive nature of the procedure would have detrimental effects on sperm viability and quality. Furthermore, the equipment expense, speed, and sorting efficiency dictated that commercial application would only be possible with extremely low sperm numbers per insemination dose.[3,4] Reduced sperm numbers and viability with sex-sorted semen results in compromised conception rates[5,6] and concerns related to the economic feasibility of the technology.[7,8] From the initial reports of Garner et al.,[1] an additional 15 to 20 years of research elapsed before the procedure was ready for commercial application in the bovine. Despite sweven years of successful commercial application, the technology still suffers most of those same limitations in sorting speed and conception rates. Although sorting speeds have been enhanced with conversion from analog to digital electronics, conception rates remain ~80% of those obtained with conventional semen. Sperm dosage trials imply that barring major technology advancements, comparable conceptions rates to conventional semen is unlikely.

Nonetheless, sexed-sorted semen is and will remain a commercial reality.[9,10] Though the product is still not recommended for use in lactating cows, producers appear willing to risk the addition loss in conception in order to produce heifers from some of the more genetically valuable member of the herds. This trend for use of sex-sorted semen in cows is especially evident in Jersey herds where cow conception rates are greater as are the economic losses associated with the birth of Jersey bull calves. Recent high prices for beef somewhat diminishes incentives for use of sex-sorted semen, but supports the trend for application in the genetically desirable cows and allow the lower end of the herd to be bred to conventional beef semen. Although male-sorted beef semen would be the more desirable application, this is not likely to occur until the issue of compromised conception with sex-sorted semen is resolved. At present, the differential value of male vs. female exclusively for beef production cannot offset the economic losses due to reduced conception rates from sorted-semen in either dairy or beef production systems. However, there is no question the use of both sex-sorted semen and the increased use of conventional beef semen are complimentary evolving technologies that are creating a new paradigm in philosophy of dairy cattle breeding.

Aside from the direct cost of sorting using a $500,000 piece of equipment that produces ~12 to 14 doses per hour, sperm sexing adds many additional expenses with respect sire management in the AI center. First, as a function of simple math of the very inefficient nature of the process, each dose of sex-sorted semen consumes resources that would have produced four or more doses of conventional semen. Thus, as more of the semen market converts from conventional to sex-sorted semen, the AI center will need more collections, bulls, stalls, labor, feedstuffs, and $$$ to produce the same total number of semen doses. In addition, the complexity of collection schedules necessary to keep a sorting laboratory supplied with product to minimize down-time for two to three shifts per day can only be fully appreciated by those directly involved. Each bull scheduled must produce a minimum number of sperm so that the sorters do not "run-out" and have people standing around in the middle of the shift. If a given collection fails to meet the minimum sperm quantity or sperm concentration standards, a back-up bull must be immediately available. Back-up bulls are the primary bulls on other days of the week, which will then need to be adjusted if they are ever called into service for back-up purposes. All this is in addition to trying to maintain adequate quantities of conventional product. With the introduction of sex-sorted semen, collection schedules in the AI center have evolved from a fixed and simple spread prepared once a week and seldom modified, to a new paradigm of a highly complex and dynamic entity this is constantly

changing and evolving requiring highly coordinated efforts across multiple departments of the AI center to effectively manage.

Will all semen sold eventually be sorted for sex? I guess I have learned to: "never say never". Technologically, sex-sorted semen is not hugely advanced from where it was 15 to 20 years ago. Price differentials among dairy calves in 2005 to 2006 (female $500 to $700: male ~$100) facilitated producer acceptance and adoption in those early years. In 2009, the industry learned the hard lesson that the true value of sex-sorted semen is less dependent on the value of heifer calves in nine months, as much as it is on milk prices and replacement values in three years. Market cycles will be constant reminders that there are limits to the economic value of sex-sorted semen. For 100% application, the cost of production and (or) the conception rates would have to eventually have to become much closer to conventional semen. This seems very unlikely using existing technology and methodology.[11] Although variant of a flow-cytometric sorting technology is underdevelopment with claims of resolution of these issues, to-date all claims are speculative based on independent patent procedures that have yet to be combined into a functional, operating prototype that can be used to validate the process and theories. Once the prototype is available, validation will likely require two years to confirm conception rates and offspring gender ratios support commercialization.

The use of a sex-specific membrane protein for selective sorting or inactivation of sperm function remains the elusive "holy grail" of all AI technologies. Although a number companies continually surface over time (much like locusts) claiming to have the answer, none have withstood the rigors of validation. Researchers this field of study have had access to >90% pure population of sex-sorted sperm for approximately 30 years now. If there were "something else" different among X and Y-bearing sperm that could be used to develop a more efficient sorting procedure, it likely would have been discovered by now. Recognizing the intercellular bridges that connect membranes of X- and Y-bearing sperm during spermatogenesis allowing for migration of any such sex-specific protein that may exist, it seems highly doubtful that such a technique has a very high probability of coming to fruition. However, it is unlikely such facts will deter the interest of those who have come to recognize that there is often; "more money to be made from milking investors than there is in milking cows".

AI submission

In the "old days", AI submission was referred to estrous detection. Although "estrous detection" is still a common term in dairy industry, what is actually performed in order to identify cows to submit for AI is typically something completely different. Somewhere during the last 20 years amidst the increasing average herd size, increasing milk production and the declining reproductive efficiency of dairy cattle, most herds have abandoned visual estrous detection as a primary method for identifying cows to be inseminated. In the western states, estrous detection has largely been replaced with once daily inspection of tail-chalk or tail-paint. With sufficient training, expertise, and facilities that promote estrus expression (dirt lots), this procedure has proven very efficacious despite limitations in accuracy and timing of insemination. This procedure allows every cow to be observed daily for the removal of chalk and when combined with the presence of secondary signs of estrus have yielded detection rates in the mid-sixty percent range.

Alternatively, frustration with all forms of estrous detection have given way to increased use of fixed-time ovulation control for both first[12] and repeat services.[13] Numerous protocols have been development, implemented, and proven effective for reproductive management dairy cattle. With adequate compliance and timely open diagnoses, pregnancy rates in excess of 20% can be routine with total elimination of estrous detection. These systems have been popular in herds located in the mid-west and eastern parts of the US where greater used of concrete and free-stall housing may negatively influence intensity and duration of estrous expression.

Although controlled ovulation programs have proven to be very effective alternatives, many producers are investigating other alternatives due to the labor, expense, and possible public perception issues associated with application of these synchronization programs. The rapidly growing alternative filling this void are automated systems to monitor estrus associated characteristics including but not

limited to mounting activity, pedometers, and more recently accelerometers that detect multiple aspect of motion activity in three dimensions. Most herds installing these systems have either maintained or enhance reproductive performance of the herd, but have done so, with dramatically reduced expense for synchronization products, which are never eliminated but rather simply more selectively used in problem females not previously identified for AI. These new paradigms for AI submission will undoubtedly continue to evolve with combinations of the above and alternative technologies that are certainly to come.

Genomic selection

Without question, the new paradigm of genomic selection promises to revolutionize the AI industry more dramatically than did frozen semen.[14] Under the traditional progeny test scenario, young sires destined for AI programs were select based on parent average. By default, full sibs were considered genetically identical even though it was well known that some would excel over others. The reliability of genetic predictions based on parent average alone was on the order of 30%. Whole genome evaluation now allows the tracking of tens to hundreds of thousands of DNA segments across generations while associating single nucleotide polymorphisms (SNPs) in individual animal with traits of economic value including but not limited to yield, health, longevity, structural correctness, and reproduction. Today all young sires entering are screened for genomic merit prior to purchase. These tests, performed at or near birth, currently provide ~70 to 75% reliability in prediction of genetic merit for yield traits, which is equivalent to 33 milking daughters under the conventional tradition progeny test scenario. This improved reliability in prediction of genetic merit young animals is rapidly impacting the industry nearly every aspect of the dairy and AI industries.

The most obvious impact of genomic selection is a transition to great acceptance and use of young sire semen by producers. Due the unproven genetic merit, young sire semen was historically viewed by producers as an inferior product to proven sires. Semen was typically sold at relatively inexpensive prices as incentive to get it used for progeny sampling, which was largely viewed as a necessary evil industry. The price/value was also often reflected in disproportionate use among repeats services, problem breeders, cows showing questionable signs of estrus. Seldom if ever did the market demand for semen from individual young sire exceed their capacity to supply product. Since the first genomic evaluations were released by USDA in January 2009, the demand young sire semen has steadily increased often far exceeding production capacity with elite genomic young sires often bearing the more premium prices of the industry.

The impact of this industry transition from mature sires to young sires will be far reaching with significant effects on semen production, semen quality control, and sire management at the AI center. First, the labor, stall space, feed, veterinary and overhead expenses are only minutely different for a young versus mature dairy sire. Yet the total number of sperm that can be obtained from a young sire in a trip to the collection arena is only 25% to 30% of his mature contemporary. Therefore, this transition dictates increased cost of semen production and more production stalls required at the AI center. Although the transition should reduce the historic stall space requirements for the three to four year rolling inventory of progeny test sires "in waiting" for a genetic evaluation, the present design of such facilities typically does not accommodate an adjoining semen collection arena. Furthermore, much of the savings associated with elimination of a large portion of the sires-in-waiting population is being offset by higher initial purchase for genetically elite dairy sires, which at present, are 5x the values of 2008. As the AI exclusivity on testing of bull calves expires in 2013 and producers begin to genomically test young sires before offering them for sale, purchase prices may increase further still and will likely result in increased interest in use of custom semen collection facilities.

All aspects of the industry (AI center and pure-bred breeders alike) are presently in a race to genetically position themselves to weather the impending storm. Although specific approaches are often highly secretive, it obvious that jumping generations is the ultimate objective. High genomic young sires and dams are being mated imminently after reaching puberty to generate the next generation for further genomic testing. In the very near future, genomically samples sons of genomically sampled sons will be available in AI production currently with the grandsire receiving his first traditional progeny evaluation.

The first milestone in consumer acceptance of this new technology is imminent with the first traditional progeny based genetic evaluations of the young sires genomically selected for sampling back in 2009. The anticipated results are expected to further drive the transition from proven sires to genomically tested young sires. Unfortunately, these results may also diminish the perceived value among producers for participation in progeny test programs that will remain necessary to continually update and calibrate the genomic predictions. Maintaining these data bases will be no small issue in today's economy where dairy producers are continually investigating opportunities to eliminate cost.

One of the greatest impacts of the genomic selection with be reflected in increased emphasis on efficiency of semen production and utilization from high demand young sires bearing one-third the production capacity of their mature counterpart. This will be discussed in detail in the following sections.

Semen quality control and quality assurance

Irrespective of the nature of the industry, any commercial enterprise with a goal of long-term profitability and success must have as a core goal the provision of a saleable product that **consistent**ly meets or exceeds customer expectations for **quality**. Of paramount importance to the AI industry are both the numbers and the quality of sperm in each AI dose. The **producer** wants assurance that the straws purchased will contain a sufficient quantity of sperm of a given quality to maximize the probability of pregnancy while costing him the least amount of money. The **AI organization** wants to sell only straws that contain a number of sperm anticipated to provide normal, acceptable levels of conception and receive a fair selling price consistent with the genetic value of a given bull. Each spermatozoon has a monetary value, dependent on purchase or lease price, genetic merit of the bull, number and quality of sperm that can be harvested, and demand for that bull's semen. Since costs of genetic and phenotypic testing (DNA and progeny analysis), bull housing and maintenance, semen collection and processing, and other items are, for the most part, similar for each bull, the number of salable doses produced per week (supply) is a major factor in determining selling price. Placing more than 'enough' sperm into an AI straw does not improve the probability of a pregnancy, but reduces number of straws available for sale. Hence, it is important to both the customer and the AI organization that number of sperm per straw is sufficient, but not excessively beyond the number needed to optimize fertility under typical field conditions. A thorough understanding of the concepts and principles of semen quality, sperm number per dose and their relationship to fertility is essential to interpreting the implications of both genomics and semen evaluation technologies on AI industry.

Principles of semen quality control

This balancing act of quantity versus quality in semen process was first described by Salisbury and Van Demark[15] and more recently reviewed in fully on text by Amann and DeJarnette.[16] For most bulls, these data fit an asymptotic curve with a gradual rise to a maximum conception rate as total number of sperm per dose increases. Both the slope of the rise and maximum value achieved differ among bulls due to the ratio of individual sperm in a sample that might be normal or abnormal because of one of two classes of defects — compensable and uncompensable. A **compensable sperm defect** is one for which conception rates improve as the total number of sperm per dose increases to a level beyond which more sperm have no benefit. Compensable defects account for the slope of the fertility response to sperm dosage. An **uncompensable sperm defect** is one for which conception rate does not change as the total number of sperm inseminated increases. The proportion of sperm in a sample with uncompensable defects establishes the maximum conception rates that will be achieved by the sire or semen sample.

These concepts are illustrated in Figure 1 for five hypothetical bulls whose semen contains different proportions of sperm with compensable or uncompensable defects. Bulls A, B, and C differ in the total numbers of sperm per dose required to achieve maximum conception rates, but not in actual observed maximum value when sufficient sperm are inseminated. Their populations of sperm have different proportions of a compensable defect(s). Note that the rate of increase in response to compensable defects is not proportionate over the entire range of sperm dosages. In contrast, Bulls D and

E cannot achieve a conception rates comparable to those of sires A, B, or C irrespective of sperm dosage, because of greater proportions of sperm with an uncompensable defect(s).

Automated semen evaluation technology

Automated systems and devices to measure numerous aspects of sperm "quality" have been implemented to various degrees across the global AI industry. When used correctly, computerized sperm motility analyzers can provide precise and repeatable estimates of sperm motility and precise descriptions of characteristics of sperm motion. Flow cytometers have been adapted to characterize numerous attributes of semen quality and sperm function based on permeability of sperm membranes to various fluorescent stains, with "leaky" membranes sometimes erroneously referred to as "dead". The Nucleocounter has proven to be a very precise technology that has largely replaced the hemocytometer for maintaining calibration of other more cost effect sperm counting technologies. In the age of automation, it is tempting to believe that the laboratory investing in the most expensive or most up-to-date technology may be the higher quality lab or be the "most correct" in their assessments. Indeed, high tech labs will often foster this 'perception'. However such may or may not be the case. It is important to recognize automated systems are NOT exempt from error of the human component. In addition, inconsistencies in sample preparation, device settings, and procedures for routine operation can influence precision and accuracy of results. **Irrespective of technology or manufacturer claims, blindly trusting a semen quality control program to a mechanical or electronic device without validation against known standards will inevitably lead to disappointment.**

The primary advantage of automation is in labor efficiency and objectivity of evaluations. Collection schedules at many major AI laboratories often approaches or exceeds 100 bulls daily. Each bull collected will have multiple ejaculates prescreened for quality prior to processing while the previous day's collections are submitted to post-quality control before transferring to saleable inventory. The manpower and attention to details required to maintain an effective program using traditional technologies becomes a daunting task. Efficiency of the semen evaluation process will be of ever increasing relevance as a greater percentage of semen sales transitions to young sires requiring more collections to produce a given number of straws.

Do automated and sophisticated technologies really provide more specific detail regarding the fertility potential of a given bull or semen sample than do traditional technologies? Perhaps, but this is not a given. It is important to remember that perturbations of spermatogenesis are typically reflected in numerous highly correlated attributes of semen quality. When one attribute of sperm quality declines, more often than not, other attributes of semen quality will likewise reflect similar declines. Thus, not only are there multiple sperm attributes that will reflect a common dysfunction of spermatogenesis, there are often numerous validated techniques to measure each individual attribute. Selection or screening samples for one attribute (whether subjective or objective) will typically enrich the retained population for other attributes as well. Screening samples for two or three different attributes greatly enhances the probability the sub-fertile samples will be appropriately identified and discarded. Provided the assessments are appropriately calibrated and based on validated associations with fertility, it is unlikely that the degree of automation or expense of procedures used really enhances the integrity of a quality control program as assessed by the semen fertility potential realized by producers purchasing the respective samples. These facts become important when considering the additive value of "new technologies". Is this truly a 'novel attribute' or are simply an alternative method to measure what we already know?

Estimates of sire fertility – commercial vs. research applications

Estimates of sire fertility provide producers with information on relative fertility potential of sires that have been used in the past and provide AI centers with feedback to monitor the adequacy of quality control and assurance programs. It is logical to assume that field fertility may indeed be the ultimate validation of semen quality. However, recognition and appreciation of the limits in both accuracy and precision of sire fertility estimates is a primary and sometimes very frustrating limitation to appropriate

use by both producers and industry personnel alike.[16] Furthermore, few outside the inner circles bovine andrologist fully appreciate the difference between commercial vs. research applications of cryopreserved sperm.

Research projects are designed to measure the impact of a change in processing procedure(s) or to relate attributes of sperm quality to fertilizing potential. In research, **variation in the attribute being measured is a prerequisite to obtaining a meaningful outcome.** In addition, the number of sperm inseminated must be below optimal levels to avoid masking a real effect by "flooding" the reproductive system with sperm. Alternatively in commercial application, the AI center **exercises the knowledge gained from research in a direct and meaningful attempt to minimize variation in fertility both within and across sires.** These objectives are accomplished by adjusting sperm numbers per dose, discarding individual collections that do not meet minimum standards and, if necessary, by culling of sires. Ultimately, a key characteristic of a sound commercial semen quality control program is the overall lack of variation in field estimates of sire fertility combined with a lack of correlation between the semen quality traits measured and field fertility. Such characteristics should be recognized as comforting confirmation that the system in place, be it more or less sophisticated than some other, is working and yielding a 'consistent quality product'. When novel attributes of semen quality are identified that do correlate with field fertility, the competent laboratory will add these traits to the list of reasons for culling collections and (or) bulls and the correlation with field fertility in the commercial setting would again evaporate.

In reality, most in vitro methods to assess semen quality are more accurate and precise than are estimates of semen fertility potential based on population data.[16] Typically, 85 to 90% of all sires from major AI centers (including progeny test sires) yield estimates of field fertility within ±2 to 3% of breed average and statistically indistinguishable from each other when service numbers, environmental noise, data errors, and binomial variation are considered. The more accurately and precisely we can estimate any attribute of semen quality that is associated with fertility, the more precisely a proficient laboratory can designate appropriate sperm dosages to ensure both optimum efficiency of utilization and optimum fertility potential of each sample. Laboratories with limited precision in semen quality assessment may use increased sperm number dosages as insurance to mask deficiencies in quality control. This often precludes the ability to detect a measureable "fertility difference" among laboratories even though a real and measureable difference in semen quality may exist nonetheless. The primary difference a producer might recognize from the more vs. lesser proficient laboratory is in semen availability of high demand sires, which in some cases will also translate into higher prices per dose based on supply and demand. These differences will become increasingly important to remember as we enter a new paradigm of with more custom collection laboratories entering the global semen supply chain. Just as it is misleading for "high tech" laboratories to imply superiority because of technology investments that may or may not necessarily yield higher fertility semen, so too are the deceptive approaches of touting high cell number dosages that seldom yields field fertility estimates that add validity to the claims.

As the global AI industry places ever increasing emphasis on fewer and fewer sires of younger and younger ages, a paradigm shift in philosophy of sire fertility evaluations is in need. Presently, most popular systems use a three or four year rolling database of inseminations to calculate estimates. In such a lengthy period, popular sires can accumulate tens to hundreds of thousands of services that diminishes the sensitivity of the evaluation to detect "recent changes" in fertility in response to changes in sperm quality and (or) cell numbers per dose. Some AI organizations have redundantly invested in sire fertility evaluations systems to satisfy this need.

The next paradigm is sire fertility evaluation will occur when electronic data capture technology harnesses the information that can be gleaned from the bar-codes already printed on straws by some organizations that indentify by sire and specific collection codes within sire. In addition to improving accuracy and efficiency of service sire recording, this marriage of already existing technologies will allow the AI centers to precisely associate field fertility with specific semen quality attributes captured in the laboratory and will hugely advance our understanding the components of male fertility.

The new regulatory paradigms

Shrouded within the technology advancements already realized by a highly competitive AI industry, is the fact that many of these advances are not fully appreciated by national and international regulatory agencies. As opposed to the early days of AI where research was openly published and shared for the benefit of all, today's major AI centers are in constant battle for survival in a highly competitive global industry. Decades of effort and research have yielded novel extenders, extender additives, cryopreservation techniques, and semen quality diagnostic procedures that are not always published for public consumption but remain in closed circles of proprietary technology for the respective AI center. This unfortunately leaves regulatory bodies of various importing/exporting countries nothing but antiquated research from decades prior upon which they attempt to place minimum standards for cell numbers and semen quality. Though well intentioned, in most instances these regulations unnecessarily impose minimum standards that negatively influence both price and availability of semen with no fertility benefit to producer. The solutions are not readily apparent but recognition of the issue is likely the first step towards resolution.

Summary

Commercial introduction of sex-sorted semen and genomic evaluations will likely change the AI and dairy industries more over the next five to 10 years than it has changed in the last 40 years. Implementation of automated technologies for semen evaluation and processing will continue to enhance laboratory labor efficiency and efficiency of semen use. This will be critically needed as more of the supply comes from sire of younger ages and lower production capacity. These technologies may also indirectly improve conception potential within some laboratories by more accurate identification of individual collections/sires that should be culled and discarded, but enhanced fertility potential of otherwise normal collections processed by competent laboratories (irrespective of degree of automation) is an unrealistic expectation from any technology. Fewer herds of larger size under ever increases economic constraints will continue to drive change and force out inefficiencies from every aspect of both the dairy and the AI industry.

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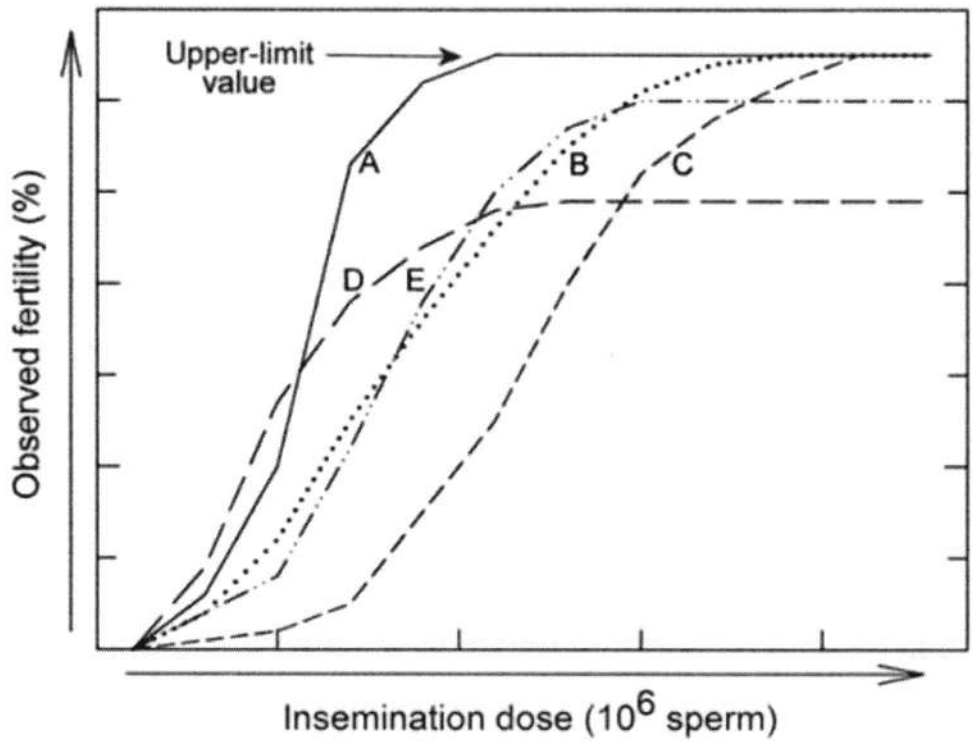


Figure. 1. Relationships between number of sperm per AI dose and fertility for five hypothetical bulls. Semen from bulls A, B or C contains few sperm with an uncompensable defect, whereas semen from bulls D or E contains a substantial proportion of sperm with an uncompensable defect. The 'upper-limit value' is an average from use of semen from a bull in many well managed herds, but might be substantially higher or lower in a given herd. In herds where the upper-limit for fertility is lower than depicted, pregnancy rates obtained with Bulls A-E will be proportionally lower.

The computer generated bull breeding soundness evaluation form—a marketing tool for theriogenologists or just something pretty to look at?

John L. Myers
Pecan Drive Veterinary Services, Vinita, OK

It was apparent by the second semester of my eighth grade year that Sue Ellen Owens was the smartest student in our class. It was obvious that while our teachers admired her a great deal she earned her high grades fairly through a combination of rapt concentration in class, innate intelligence and attention to detail. One of these details was her consistent placement of our English assignments done at home in an attractive binder that distinguished her papers from everyone else's and surely caught the attention of the teacher, the rest of the class and especially me.

I cannot help thinking of Sue Ellen today as I introduce the Society for Theriogenology computer generated and online Bull Breeding Soundness Evaluation (BSE) form. I am sure that the English homework she produced in the eighth grade would have scored just as high a grade had she turned them in without a binder—just as surely as my work justified its same sub-Sue Ellen result even when I imitated her technique of presenting my themes in an over-the-top folder my father used to present his engineering work—but I wonder when this new form is used are will we be promoting the practice of quality soundness examinations, establishing the standards by which bulls should be measured, encouraging the use of a common, efficient, recognizable and attractive form that will be embraced by a large number of veterinarians and providing a method by which measurements can be learned or enhanced, or are we merely providing to the veterinary consuming public the same uneven and sub-standard work placed in an pretty plastic binder?

Keywords: Breeding soundness evaluation, computerized form, electronic medical record, Society for Theriogenology

Directive to produce the computer generated BSE form

The machinations of committee work as told through reports or minutes surpass in boredom a thoughtful reading of the Internal Revenue Service code. From the perspective of the committee itself, however, embarking on modification of the existing BSE form became an adventure that changed from what we thought was a concept of a fill-in-the-blank spreadsheet exercise to a full-fledged immersion into the bizarre and mysterious world of program writing. Along the way the committee had serious and long discussions on graphic artistry, color combinations, pathology prevalence, confidentiality, keystroke shortcuts, economic impact to the Society for Theriogenology (SFT) and the most efficient process in making our wishes known to the programmer.

Dr. Richard Hopper, then president of the SFT, gave the directive at the winter meeting of the 2011 SFT board in Milwaukee, WI to develop a computer-generated BSE (CG-BSE) form for bulls. Assigned to the committee were Drs. John L. Myers, Herris Maxwell and Will Shultz. Dr. Shultz had commissioned a company to design a program for tracking and identification of frozen canine semen and recommended the same company be retained for development of this initiative. Soon thereafter Drs. Michael Thompson and Brian Keith Whitlock were added to the committee as well as the SFT executive director, Dr. Charles Franz.

Between the January of 2011 SFT board meeting and the subsequent SFT convention in August of the same year the committee worked to develop a temporary model of the CG-BSE form to be displayed for comments and suggestions at both the August SFT meeting and later the next month at the American Association of Bovine Practitioners (AABP) convention. To describe the product on display at the two conventions as a BSE form would be similar to saying since a six-grader can read he's ready for college.

Many useful comments were garnered, especially at the SFT convention. Perhaps the most useful response from the AABP would have been the general feeling of apathy to any sort of BSE form produced by the SFT because individual clinics and veterinarians developed their own forms separate and

distinct from any other entities and not necessarily conforming to standards set by the SFT or any other organization. As an aside, the programmer developing the CG-BSE form was given money for travel and lodging to attend the AABP convention to hear first-hand any comments or suggestions.

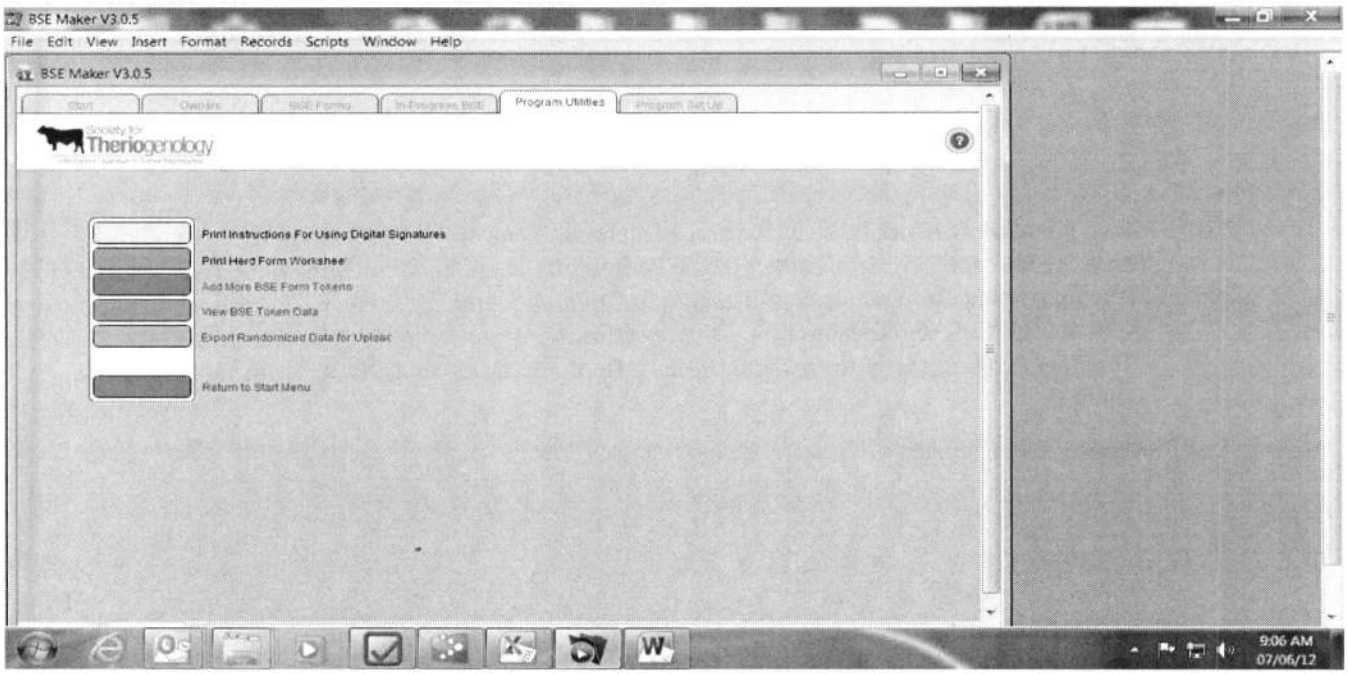
Shortly after the AABP convention progress on the CG-BSE form stopped. Through negotiations between the SFT executive director, Dr. Charles Franz, and the programmer's superior it was decided that either the SFT and the company doing the programming would abandon the project with a full monetary refund, or the company would need a great deal more money to move forward. Through the counsel of Dr. Franz, the committee decided to take the refund, sever ties with the programming company and look elsewhere for help in completing the project. Once again, Dr. Will Shultz had a prospect.

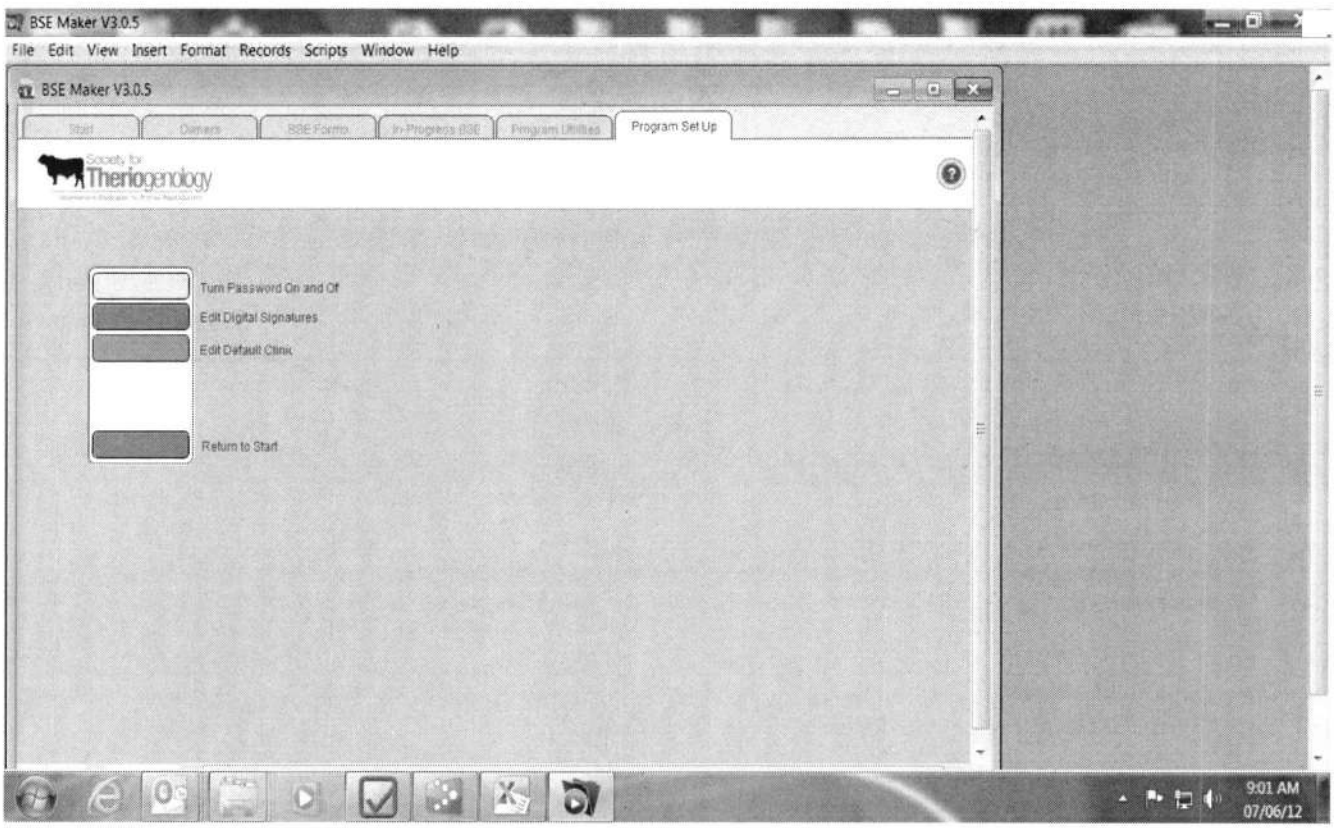
The programmer Dr. Shultz suggested was David Riedle of Riedle Consulting and he is the person responsible for the form as it now presented. The relationship between Dave and those working on this project has been excellent, and while Dave seems to enjoy working with us we have no idea how he would assess our committee about cooperation, quality of direction or understanding of the concepts underlying computer programming. From the committee side of the equation, however, we all believe that if it is possible for a computer by means of a program to do anything at all, Dave Reidle can make it happen.

This brings up the seductive elements of program writing experienced by the committee once we began working on the project. We realized that magical feeling that movie makers must feel when they imagine Mark Ruffalo's muscles enlarging so much as he turns into the Great Hulk that the seams burst on his clothes as he climbs a building and throws a bus at someone evil. Similarly, we now possessed the possibility that we could design a form that could streamline the process of completion, feature accoutrements that visually enhanced the professionalism and attractiveness of the form, provide for accumulation of a large amount of data, encourage careful and complete examinations of the bulls being tested and maintain an efficient method of continuing a revenue stream for the SFT. The committee established priorities that insured the delivery of the features listed above while preventing the allure of making our form turn green and throw busses.

Principles guiding the development of the CG-BSE form

1. The guidelines and standards previously set by the SFT would be maintained in the new form.
2. The CG-BSE formwould not only be easy to use, but would require less time to complete than filling out the paper form by hand. Through the use of a digital signature, a repeatable method in memory to provide information on the veterinary clinic on each form, an efficient method of quickly finding owner information that can be placed instantly into the proper fields, and several drop-down menus that provide appropriate information with the click of a mouse has produced a product that can be completed very quickly. Those familiar with the program can easily complete the form in thirty seconds or less.





3. The CG-BSE form would be capable of producing an abbreviated form which lists the classification of the bull without revealing specific defects or motility as well as the conventional

form that would list all specifics. Some members of the committee have witnessed confusion in instances when a bull buyer, unfamiliar with sperm defects, displays concern over any sperm defects regardless if those numbers are within the limits of a satisfactory classification. When the particular bull's specific data are uploaded into the centralized database, however, the specifics remain intact even if the form did not display them.

4. The CG-BSE form would be attractive, professional and difficult to reproduce by copying. The paper form of the BSE has a picture of Nandi which is not conducive to use in the computer form. Because of the affection for Nandi among influential members in the SFT, the committee spent a great deal of time and discussion on the substitution of a silhouette of a generic bull into the logo. Computers can do many things, but in this case it could not keep Nandi from looking like a smudge.

Society for Theriogenology

Bull Breeding Soundness Evaluation

Guidelines Established by Society for Theriogenology
P.O. Box 3007 - Montgomery, AL 36109
Phone (334) 395-4666 - Fax (334) 3399 - www.therio.org

JOHN DOE FARMS
JOHN DOE
222 NORTH ROAD
VINITA, OK 74423
(918) 232-1282

BSE Date: 7/6/2012	BSE Case No: 2187-1
Bull Name: CHAMP	Breed: LIMOUSIN
Bull I.D. No: Y11	Brand ☐ Tattoo ■ Ear Tag ☐
Bull Birth Date: 12/25/2010	Age (Mo.) 18

PHYSICAL EXAMINATION

Body Condition Score: Beef - 6
Thin ☐ Moderate ☐ Good ■ Obese ☐
Pelvic Ht. Pelvic Width Pelvic Area

Feet/Legs	■
Eyes	■ SPOT IN LEFT EYE
Vesicular Glands	■
Ampullae/Prostate	■
Inguinal Rings	■
Penis/Prepuce	■
Testes/Spermatic Cord	■
Epididymides	■
Scrotum (Shape)	■
Other	
SCROTAL CIRCUMFERENCE (CM)	36 . 0

SEMEN EXAMINATION

Collection Method: EE ■ AV ☐ Massage ☐
Response: Erection ■ Protrusion ■ Ejaculation ■

Semen Charicteristics		Ejaculate 1	Ejaculate 2
Motility	Gross	60	
	Individual (%)		
% Normal Cells		92	
% Primary Abnormalities		5	
% Secondary Abnormalities		3	
WBC, RBC, Other			

CLASSIFICATION

Interpretation of data resulting from this examination would indicate that on this date, this bull is a:

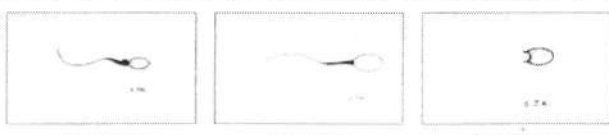
Satisfactory Potential Breeder

Re-examination recomended on:

This bull has been examined for physical soundness and quality of semen only. Unless otherwise noted, no diagnostic tests were undertaken for libido, mating ability, or infectious disease status of this bull.

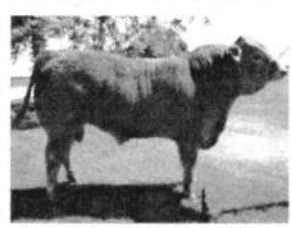
Signed: John L. Myers D.V.M.
Member - Society for Theriogenology

Pecan Drive Veterinary Services
P.O. Box 463
Vinita, OK 74301, OK 74301
(918) 256-7803



Proximal Droplet Macrocephalic Detached Head

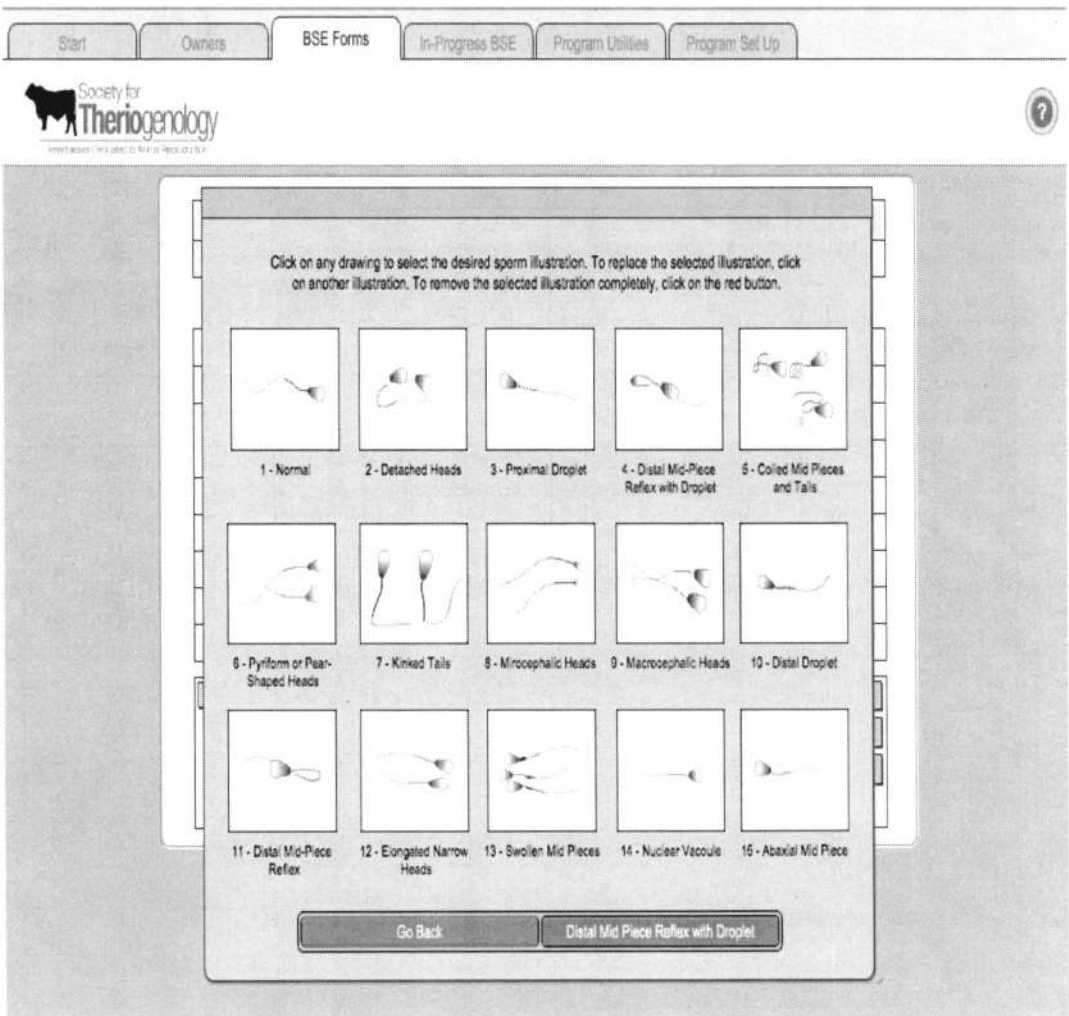
Remarks and interpretation (diagnosis, prognosis, recommendations)



Colors were used to highlight two different items: The SFT logo and the classification of the bull. The committee felt that the color in the logo (SFT Green-Pantone 363 green) is good marketing for the SFT and the red, yellow and green in the classification (signifying in order Unsatisfactory, Deferred and Satisfactory) lent not only an immediate and familiar assessment of the examination but further provided gravity for the reason the bull was tested in the first place.

Finally, it is not uncommon for veterinary practitioners to fabricate their own BSE form by copying the current paper form and performing modifications such as removing the SFT logo and inserting the veterinarian's name and clinic. This not only is a violation of copyright but deprives the SFT of revenue to which it is justifiably entitled. The CG-BSE form makes it difficult to perform those types of sinister behaviors.

5. The CG-BSE form would encourage careful and complete examination of the bulls. One feature of the new form is its inclusion of a drop-down menu of fourteen common sperm defects as well as an illustration of a normal sperm.



The committee believes this offers a great marketing tool for the veterinarian, in that he or she can, by means of several illustrations, convey to the client what was discovered while examining the morphology stain. While it is not a requirement for completion of the form that the illustrations be included, this will be one method that will enforce to perception of the

differences between those veterinarians who do a complete evaluation from those who pronounce a bull satisfactory from a cursory examination and a quick look at motility.

Further, we believe the illustrations not only provide a guide for those new to the BSE business to see defects that are commonly seen, but it also provides a means—limited though it may be—to look for persistence in specific defects in subsequent examinations.

6. The use of the CG-BSE form should make economic sense for both the SFT and the users of the product. The sales of the paper form of the bull BSE form produce an annual income that while not large is nonetheless important, and it is hoped that the new CG-BSE form will gain equal or greater acceptance. There is no plan to discontinue the paper form of the SFT BSE form. Just as paper forms are now sold in booklet fashion by the SFT office, the CG-BSE forms will be purchased by tokens. There will be a tab within the program that, upon opening, will give access to purchase a batch of forms through use of a credit card online transaction. Additionally, there is a mechanism that will alert the user to the number of forms yet unused, and we think that this will not only streamline the access to SFT BSE forms but in fact creates a greener, paperless solution to the BSE.

   Currently the committee has considered a price structure for the program download that includes 50 forms. The program purchase is a one-time event which includes support, and subsequently the forms will be sold in the form of tokens at $0.25 apiece. The current price to purchase one unit of the paper BSE form is $0.33 but when subtracting the cost to print the form the gross net income to the SFT is greater when the computer generated form is used rather than the paper form.

7. The records for each bull examined will stay within the program under the owner's account. Revisions can be made to any part of the CG-BSE form up until the record is marked as complete, and at that time the token for that form is spent and changes can no longer be made. The form (either the long or the short form) can be printed on the completed form, however, as many times as needed or desired.

   While our particular clinic will never be nominated for the Model of Efficiency Award (should there be one) we will examine around 1000 bulls a year and we are perpetually looking into files in pursuit of our findings on a bull we examined previously. Since we retain the yellow copy of the paper form in our files we must thumb through several sheets of paper to find the record of our previous examination. Many times that is successful if we can find the owner's file and if we have filed the paperwork properly. Since instituting the CG-BSE form we have been able to recover needed records of previous examinations relatively effortlessly although we still print a copy of the examination record and place it in the owner's file if we can find it. From a personal viewpoint, retaining the examination records on a computer and unlimited printing of the form have been some of the more surprising and valuable features of this program.

8. Finally, within this program is the ability to accumulate a large amount of information in a centralized location. Once again, a tab exists that takes one through the process of uploading data, and we believe it is as simple, intuitive and safe as uploading other information (accounting, brucellosis procedures, etc.) that we already perform. We do not presume that all of the data will be of high quality, but the committee does imagine the possibility of a large quantity. While I understand the repulsion those scientists among us have to large amounts of questionable information, the committee believes this is an avenue by which the quality of the BSE's of bulls can be elevated. If an individual practitioner can compare his culling percentage to other practitioners, his evaluation of his own methods may cause him to change for the better. Comparing number of abnormal sperm or scrotal circumferences among breeds, ages and locations of bulls would be of great interest, but only in numbers large enough to have confidence that the trends seen are valid. The committee feels that from data collected from this program

already and the anticipated percentage of those who would buy based on practitioners already using the paper form, information from 10,000 to 20,000 bulls a year would not be unreasonable.

Discussion

In March of this year David Riedle rolled out the first version of the CG-BSE form. This form was designed for a computer but not an iPad, and there were four users: Herris Maxwell (Auburn University), Brian Keith Whitlock (University of Tennessee), Mike Thompson (Willow Bend Animal Clinic, Holly Springs, MS) and John L. Myers (Pecan Drive Veterinary Services, Vinita, OK). At the time that we cut off uploading results into the program to accommodate a deadline we failed to meet several times, there were 634 records in the database.

The classification of the 634 bulls is as follows: Satisfactory: 529 (83.4%), Deferred: 48 (7.5%), Unsatisfactory: 57 (8.9%).

Following are the breeds and their Satisfactory Classification rate:

A. Angus: 477 (75.2%), 82.6% classified as satisfactory
B. Simmental: 26 (4.1%), 92.3% classified as satisfactory
C. Hereford: 25 (3.9%), 84% classified as satisfactory
D. Red Angus: 20 (3.2%), 85% classified as satisfactory
E. Charolais: 18 (2.8%), 77.8% classified as satisfactory
F. Brangus: 11 (1.7%), 90.9% classified as satisfactory
G. Gelbvieh: 9 (1.4%), 100% classified as satisfactory
H. Limousin: 6 (0.9%), 100% classified as satisfactory
I. Other Breeds: 40 (6.3%), 80% classified as satisfactory (other breeds: Sim/Ang, Angus Cross, Black, Brahman, Limiflex, Ultrablack, Balancer, Beefmaster, Bucking Bull, Longhorn)

The committee is proud of the great amount of work to get this number of records into a database and we realize that the numbers represented still do not in many cases constitute a large enough volume to draw valid conclusions, although there would certainly be Limosin breeders who would willingly declare that in over 633 bulls, tested the Limousin breed was found to be 100% fertile. The bulls' 83.4% Satisfactory Classification does, however, fit within the wide range of previous reports.[1-3] We believe with coordination and cooperation with those competent to analyze data and suggest corrections and improvements we have the possibility of producing an ongoing large amount of data that could result in higher quality and more accurate examinations of bulls for breeding soundness.

There is now a version of the program compatible with the iPad.

Conclusion

There has been a serious amount of work and cooperation to produce the CG-BSE form presented today. While we hope the points made above as to the principles the BSE committee used are valid and convincing as presented, we would like to conclude with the idea that this form could be used as a marketing tool for the following:

1. A private clinic or practitioner
2. A university clinic
3. The importance of Breeding Soundness Examinations
4. The Society for Theriogenology

The origins of the Society began with a few interested and dedicated individuals who were involved with bull fertility. The form as we present it today represents but a blip in the progress and expansion of a field that encompasses more species, procedures and knowledge than the gentlemen in 1954 would have ever imagined. With that history the committee not only feels gratitude but more importantly responsibility.

I began by relating my impressions of an eighth grade girl who turned in her assignments in a pretty plastic binder. Sue Ellen moved away in the ninth grade but I still remember how attractive and intelligent she was. The binders into which she placed her work were no guarantee that what was inside was of high quality, but it became clear that the binder was evidence that she took pride and care with what she produced. The CG-BSE form's usage also does not guarantee high quality work, but it does represent one way to make what is done accessible, attractive and memorable.

We feel that the form represents yet another step in presenting the knowledge and work that has gone before us and it is our hope that with its acceptance we can help in the progress and continued excellence of the SFT.

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Management of urogenital injury and disease in the bull: the scrotum and its contents

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Abstract

Restorative surgery on the reproductive tract can be performed in the practice setting utilizing correctly administered regional analgesia and sedation. With respect to injuries and conditions involving the scrotum and testes, diagnosis and assignment of a valid prognosis can be facilitated by the use of several tools including history, physical examination with an emphasis on palpation, ultrasound, radiology, thermography, and the spermiogram. Surgical techniques and management tips are described.

Keywords: Bull, pudendal nerve block, hemi-castration, scrotum, testicle

Introduction

Surgical and medical management of injuries and other problems of the scrotum and the testes are of value only if they result in restoration of fertility. Additionally, economic considerations are paramount and the ability of our clients to make informed decisions is dependent on both a valid prognosis and a cost effective treatment, which in turn are dependent on effective diagnostics and practical surgical remedies.

Most maladies of the scrotum and its contents can be effectively diagnosed with a good physical examination. Adjunct diagnostic modalities such as ultrasound (+/- Doppler), radiology, and thermography can provide additional information that further refines the diagnosis and more importantly the prognosis. Evaluation of the spermiogram can provide clues with reference to the duration of the insult and potentially the prognosis.

Regional anesthesia techniques that can be utilized for surgeries of the scrotum, as well as penis and prepuce will be described. The combination of sedation and regional anesthesia allows these surgeries to be performed on a standard hydraulic or manual (tilt) table.

Following successful surgical intervention, most bulls can return to service within two to three months. Most of the pathology that is seen is identified either late in the breeding season or after bulls are removed from the herd at the end of breeding season allowing the recovery time to coincide with the bull's down-time.

Conditions of the scrotum and its contents

Testicular injury

Trauma to the scrotum often results in rupture of the vaginal tunic, which leads to hemorrhage, swelling and permanent damage to the testicle. Diagnosis is typically straightforward. Palpation of the affected and unaffected testes will often lead to the diagnosis, but the use of ultrasound and thermography can aid in establishing the prognosis. Cases that present shortly after injury may not have had significant changes to the spermiogram yet and cryopreservation of semen can be attempted for a short period of time. Studies in which scrotal insulation is applied reveal that spermatogenesis is altered within hours[1-3] of a thermal insult and abnormally high temperatures as measured by thermography will persist for at least three weeks after a testicle has been removed.[4]

In general the primary goal of management is based on salvaging the function of the contralateral testicle. To this end, the thermoregulatory process of the contralateral testicle must be preserved. Chronic thermal insult results in testicular degeneration, so surgical removal (hemi-castration) should be performed as soon as reasonably possible. However, the administration of antibiotics and a non0steroidal anti-inflammatory drug (NSAID) can be initiated as soon as the diagnosis is made, while awaiting surgery. Cold water hydrotherapy may also be helpful in decreasing damage to contralateral testicle while the bull awaits surgery and during the post surgical recovery period.[5]

Inguinal hernia

Inguinal hernias have been classified in the veterinary literature as either direct or indirect. This designation appears to have been borrowed from human literature. In the human classification an indirect hernia is one that passes through the inguinal ring and is considered to be a congenital defect.[6,7] The direct hernia is a muscular wall defect and is usually acquired. Additionally, the hernia's relationship to the inferior epigastric artery was the actual basis for classification.[8] All inguinal hernias in the bull pass through the inguinal ring,[9] (even those in which the ring is torn and enlarged) with the main differentiation appearing to be whether or not the hernia is contained within the parietal vaginal tunic (indirect hernia) or not (direct hernia). Because direct herniation involves a tear in the vaginal tunic, intestinal strangulation and incarceration are more likely and thus require immediate surgical correction as soon as the patient is stable for surgery. To further complicate our current classification, some direct hernias extend down into the scrotum and are sometimes termed scrotal hernias. Because indirect herniation is contained within the vaginal tunic and the vast majority do not have incarcerated or strangulated bowel, surgical correction can be considered elective. It might be appropriate to consider a classification change that would either be based on severity or be more consistent from a terminology standpoint.

From a diagnostic standpoint these cases will present in one of two ways; the colicky, metabolically compromised, and painful bull with strangulated, incarcerated bowel and an obvious swollen scrotum or the bull that appears clinically normal except for a swelling in the neck of the scrotum (the "hourglass" appearance).

Jersey bull with "indirect" hernia of 1 year duration.

Examination should include palpation and ultrasound of the scrotal contents along with rectal palpation of the inguinal rings. Ultrasound is especially valuable to determine the presence of bowel.

In general, both types of herniations occur primarily in mature bulls, most often on the left side and are acquired. Etiologies range from fighting to fence jumping. The most likely predisposing factor is the over-fit bull that has subsequently lost weight and now has a more assessable inguinal canal. Speculation as to why left-sided hernias are more common centers around the bull's predisposition to lying sternal-right. In the case of the typical, non-life-threatening herniation, the primary problem is disruption of thermoregulation of the testes. As described later, there are surgical options that allow for the preservation of both testicles. When there is incarceration/strangulation of the bowel preparations should be made for the administration of intravenous fluids and emergency surgery including the likelihood of intestinal resection and possible hemi-castration, and continued post-surgical care.

Hydrocele, varicocele, and scrotal fluid accumulation

A hydrocele is basically a fluid accumulation in the scrotum and virtually always within the parietal vaginal tunic.[10] Because there is communication between the peritoneal cavity and the vaginal tunic, fluid can accumulate anytime there is increased abdominal fluid (ascites). Various conditions can cause ascites and in turn scrotal fluid accumulations. A common example is hypoalbuminemia due to intestinal parasitism[11] or anemia due to *Eperythrozoon* infection.[12] In these cases the fluid is generalized (entire scrotum is enlarged). Also, *Setaria* or potentially the fibrosis suspected to be caused by their presence, has also been incriminated as a cause of hydroceles. Injury is also a possible etiology. In any case the presence of fluid has a detrimental effect on the testes, due to loss of thermoregulatory function, and thus fertility. Fertility can be restored if corrected early before testicular degeneration occurs. A varicocele is fluid or swelling within the pampiniform plexus and is usually associated with poor fertility in older bulls.[13] Varioceles are usually found when performing an ultrasound scan to investigate the declining fertility of an older bull.

Treatment of scrotal fluid accumulation due to ascites is aimed at correcting the initiating cause. For the hydrocele that is unilateral and chronic, hemi-castration of the affected testicle is a viable option.

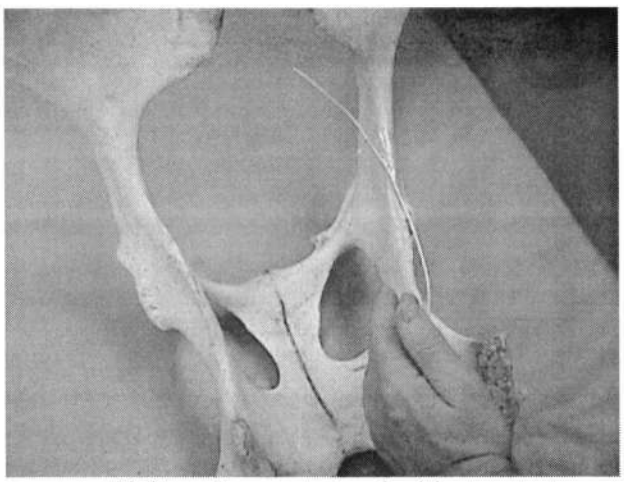
Anesthetic considerations

Although there is a strong argument that a majority of the surgical procedures aimed at restoring reproductive function in the bull are best performed utilizing general anesthesia, utilization of regional nerve blocks and sedation, coupled with good restraint is both adequate and more economic.

A suggested protocol that should be followed is to stall the bull, withholding all feed for 48 hours and water for at least 12 hours. Then in preparation for surgery the bull is sedated. The dosage is dependent on the bull's behavior, but typically 10 mg of acepromazine IV (tail vein) is adequate. The goal is for the bull to be relaxed, but he must remain standing. Two regional nerve blocks will be described, the pudendal and the sacral paravertebral. The authors utilize the pudendal for bulls and the sacral paravertebral more for cows. The bull now slightly sedated and in a chute or location from which he can be easily moved to the table is prepared for the pudendal block.

Pudendal nerve block

To perform this block, first prepare the area of the ischiorectal fossa. Then by rectal palpation locate the lesser sciatic foramen which is located wrist deep and lateral. The lesser sciatic foramen is formed by the sacrosciatic ligament dorsally and the lesser sciatic notch ventrally. The pudendal nerve is located very close to this foramen. Following the intradermal injection of a small amount of lidocaine, a 14-gauge needle is placed through the skin at the ischiorectal fossa. A 6 to 8 inch 18-gauge needle then is passed through the 14-gauge needle and with guidance from a hand within the rectum the tip of the needle is placed in close approximation to the internal pudendal nerve. Then 20 to 75 cc of lidocaine is injected. Since these are paired nerves, swap hands and sides and repeat the procedure on the contralateral nerve.

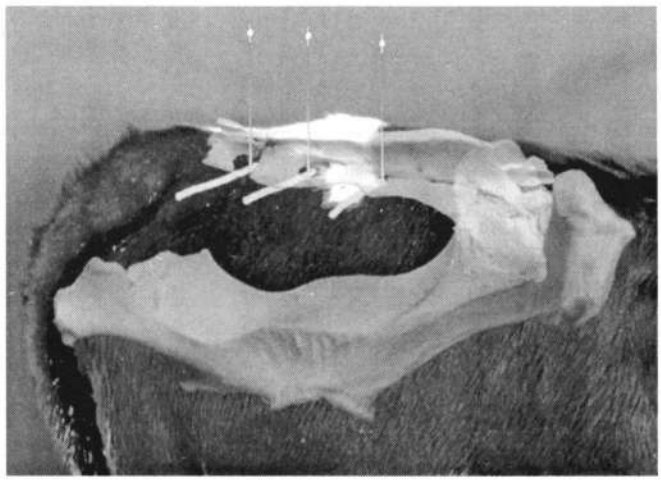


Yellow wire represents pudendal nerve

Effectiveness of this block can be evaluated by "pinching" the tail of the epididymis. The normal involuntary reaction is a lifting of the testicle. Inability to lift or retract the testicle signifies adequate analgesia. Since each testicle receives independent innervation both epididymides are tested.

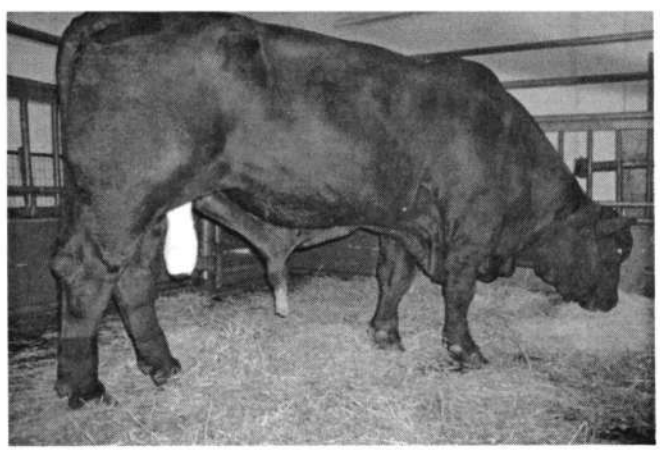
Sacral paravertebral nerve block

Blocking the pudendal, middle hemorrhoidal, and caudal hemorrhoidal nerves is easily facilitated by blocking S-3, S-4, and S-5 as they branch off the spinal cord, the important anatomical landmarks are the foraminae that are located lateral to the dorsal midline. Because lateral movement increases the difficulty, squeeze chute restraint and tranquilization is recommended. A caudal epidural may be beneficial in the flighty or hypersensitive individual as well. Clip and perform a surgical preparation of the skin over the dorsal sacrum. The paired S-5 foramina are 1-2 cm lateral to the sacral-coccygeal joint (the most cranial of the joints movable when the tail is raised and lowered). The S-4 foramina are about 3-4 cm cranial, but more lateral and the S-3 foramina are an additional 3-4 cm cranial. A stab incision dorsal to each foramen will facilitate the introduction a 5-7 cm, 18 gauge needle. These foramina can be palpated rectally and a finger placed in or over the ring both identifies the structure and assures the correct placement of the needle. When the osseous ring is entered inject 2-3 cc of lidocaine or an alcohol/lidocaine mixture if the purpose of the block is to manage tenesmus following such conditions as rectal prolapse or a chronic vaginocervical prolapse. An effective mixture is 1 cc of 2% lidocaine and 2 cc of 95% ethyl alcohol. This mixture should effectively decrease tenesmus, while maintaining tail viability



Hemi-castration

The technique for hemi- or unilateral castration is straightforward and can definitely be performed in the bull with a combination of sedation, good restraint and regional or local anesthesia. The bull should be fasted for 48 hours and water withheld overnight. Antibiotics are begun the day before surgery and continued for at least five days after surgery. A pudendal nerve block can be performed on the ipsilateral side to the affected testicle or a local anesthetic can be injected into the neck of the scrotum, infiltrating the spermatic cord.[14] The surgical area is prepared for aseptic surgery as the scrotum will be closed. An elliptical incision the length of the testicle, taking care to leave the parietal tunic intact, will provide excellent exposure and serve to minimize dead space following removal of the testicle and closure. After blunt dissection to free the testicle the parietal tunic is excised. This exposes the testicle and the spermatic cord. The cremaster muscle is transected and the spermatic artery and vein are double ligated with an absorbable suture (#0 or larger). Remove most of the parietal tunic, leaving enough to suture over the cord stump. The cord can be closed with #0 absorbable suture as well. Close the dead space and place a Penrose drain that exits at the most distal aspect of the scrotum, two to three cm from the distal end of the incision. The incision can then be closed with #2 non-absorbable suture material utilizing a suture pattern of the surgeon's choice. The authors then suggest wrapping the scrotum containing the remaining testicle with elastic tape.



This is especially useful if the bull will be stalled for a few days and exercise is limited. The wrap is removed in two days and the Penrose drain is removed the following day, but can be left until day four after surgery. As stated previously hydrotherapy can be utilized if significant swelling occurs, but two days with the aforementioned bandage and a return to the pasture seems to be the best way to avoid swelling.

Inguinal hernia repair

The two approaches to the repair of an inguinal hernia include a standing, flank approach utilizing regional anesthesia and an inguinal approach in which the bull is placed in lateral recumbency- affected side up.[9] The standing, flank approach is the definite choice of the authors if the scrotal contents can be moved during palpation of the scrotum and testes. Additionally, in the case of an elective repair of an indirect inguinal hernia, some hernias will, following a 48 hour fast, become less evident externally as abdominal contents exert less pressure. However some hernias cannot be repaired via this approach and an inguinal approach has to be used.

For a standing flank approach, the ipsilateral paralumbar fossa is prepared for surgery. An inverted-L local or paralumbar block can be employed. After entering the abdomen, the inguinal ring is identified, the herniated tissue grasped, and with traction applied, the hernia is reduced. Adhesions to the parietal vaginal tunic or from fibrosis due to the tearing of the tunic complicate this and may necessitate an inguinal approach. However, if the hernia can be reduced, the inguinal ring closed with a blindly placed suture. One of the authors (Hopper) utilizes a continuous "blind" suture pattern in which a length of suture material (#2 chromic catgut) is passed through a needle, doubled, and a knot tied about 12-15 cm from the end (tail). After the first pass through the tissue, the needle is passed back through and between the doubled suture, pulled tight (cinched), and continued. When the end of the ring is reached, go back the other direction and then tie to the "tail". Thus, only one knot is needed. Care to avoid regional vasculature is of obvious importance. The flank is closed in a routine manner.

The inguinal approach for inguinal hernia repair necessitates the use of general anesthesia. The bull must be in lateral recumbency with the affected side up. The upper rear leg must be raised and the inguinal area preppared. A 20-25cm incision is made over the inguinal ring and continuing through the subcutaneous tissues approach, identify, and expose the spermatic cord. In the case of the direct hernia, you will have likely already encountered herniated bowel and identification of non-viable bowel and resection/anastomosis will be the next step. If the parietal tunic is intact, the spermatic cord guides blunt dissection toward the ring. The tunic is then incised to facilitate the examination of the hernia contents and specifically the presence of adhesions. Blunt dissection to separate the adhesions is performed only after pre-placement of one or more ligatures. Reduce the hernia contents and then identify and remove the inguinal fat pad if present. Closure of the ring is a two step process.[9] First, using #4 non-absorbable suture and an atraumatic "hernia" needle take a "bite" 2 cm from the medial aspect of the ring, going on one side of the spermatic cord engaging the internal abdominal oblique muscle and then returning on the same side go back through close to the original "bite" (do not tie). Then repeat on the other side of the cord, making sure that the point engaged on the external abdominal oblique is at least 3 cm from the first. Tighten and tie both at the same time. Pulling the muscle over adds stability to the closure and "protects" the cord. The ring is then closed with an overlapping pattern suture pattern again utilizing non-absorbable suture. Close the ring down to the point where two fingers can still be placed adjacent to the cord. After closing the incision that was made in the parietal tunic as well as the dead space the skin is then closed with a Ford inter-locking pattern.[9]

Regardless which procedure is employed, antibiotics are continued for five more days and as in the case of the hemi-castration return to a small lot from a stall provides the exercise that will help to minimize post-operative swelling.

Conclusion

Surgical procedures that can potentially restore the fertility of the bull should increase in demand with the recent increases in cattle prices and specifically the sale prices at auction for elite breeding bull

prospects. A careful diagnostic approach that assures a realistic prognosis is essential for producers making an economic decision regarding an injured bull. Use of regional anesthetics instead of general anesthesia, when possible and acceptable can also make many of these restorative surgeries more economical.

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

Selected surgical conditions of the bovine penis and prepuce

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Abstract

Any abnormality that impinges on the bull's ability to achieve either erection and extension of the penis or insertion and penetration of the vagina results in immediate infertility. Abnormalities may be congenital: persistent frenulum, micro-vascular shunts of the corpus cavernosum penis; infectious: fibropapilloma; or acquired: penile hematoma, preputial prolapse, phimosis, paraphimosis, hair rings, etc. Some conditions such as penile deviations may be developmental in nature or result from injury. Repair in turn may be relatively simple such as transecting a frenulum tag or removing a single wart. Severely damaged preputial tissue resulting in either phimosis or conversely paraphimosis with extensive damage to preputial tissue may require lengthy hospitalization and aggressive surgical intervention. Additionally, provision of an accurate prognosis and reliable cost estimate is critical to the prospective consumer (client) of these services.

Keywords: Bull, penis, prepuce, injury, hematoma, prolapse, surgery

Introduction

With recent increases in the value of breeding bulls, procedures both medical and surgical that are aimed at restoring breeding function should increase in demand. Economics, both the value of elite genetic bulls and the costs of providing surgical and medical treatments are factors in the demand for these techniques. Phenotype and breed popularity likewise impact demand as many surgical conditions have breed/type predisposition. Virtually all of the restorative surgery techniques utilized today for injuries and conditions affecting the penis and prepuce of the bull were first described over 30 years ago.[1] These procedures, any updates or improvements, as well as cost saving measures will be the focus of this paper.

Persistent frenulum

Separation of the skin that connects the penis to the prepuce begins around four months of age in bull calves and should be complete by one year of age. Partial or complete failure of separation results in an inability to extend the penis. Individuals with Brahman influence may however have enough redundant preputial tissue that extension is possible, but these must be corrected as well because this tissue is extremely prone to injury. In the vast majority of cases diagnosis is made during the breeding soundness examination (BSE) and this is of course a reason that full extension of the penis allowing complete visualization is a crucial aspect of a correctly performed BSE. This also represents an appropriate time to correct this abnormality and in fact some will tear when the penis is extended. Correction is straightforward. Simply transect the tissue band, utilizing hemostats or sutures if needed.

Fibropapilloma

Fibropapilloma or warts are a common finding in young cattle under the age of 24 months. Group housing of young bulls along with a predisposition for homosexual behavior both increase the incidence and add to the possibility that the penis will be among the affected locations. Penile warts are usually identified at the time of breeding soundness evaluation[2] and again underscore the importance of visualizing the penis during semen collection. Alternatively, young bulls that are reluctant or unable to breed may be affected. Bulls may present with either phimosis or paraphimosis.

Careful surgical removal whether it be by excision, cryotherapy, use of a laser, alone or in conjunction with immunization are possible management modalities. Immunization can be accomplished with the use of either a commercial or prepared autogenous wart vaccine. For many years one of the authors (Hopper) recommended the use of a commercial wart vaccine for clients with bulls being prepared for shipment to bull test stations where they would be housed with multiple bulls from multiple

sources. Additionally, this author found the use of an autogenous vaccine to be of value in herds that were experiencing a high incidence. A description of the preparation of autogenous wart vaccine is included as an addendum.

Hair rings

Less common than warts, the presence of a hair ring is likewise due to homosexual behavior and group housing of young bulls. Accumulation of hair during riding and the continuous action of riding, extension and retraction of the penis serves to create a tight band of hair. These are typically identified during a BSE and treatment is simply removal and application of a suitable ointment. Careful examination to determine the depth of the lesion and possible damage to the urethra[2] is warranted. Small fistulae will typically heal, but those that are larger, more proximally located should be closed.

Preputial injury

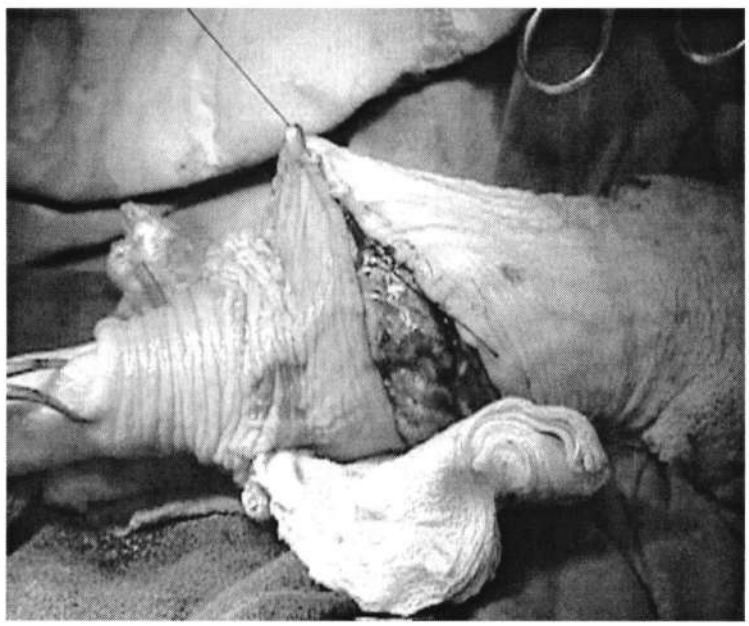
Preputial prolapse is alternatively a predisposing factor or result of trauma and bruising of the prepuce. It is more common in the Brahman influenced breeds due to their pendulous sheath, redundant preputial tissue, and larger preputial orifice.[2-5] Polled breeds typically lack the preputial retractor muscle thus exacerbating the condition when combining genetics with the Brahman. Minor trauma with prolapse (Grade 1) can be treated with various medications and wrapped. More extensive trauma with swelling and the presence of necrotic tissue (Grades 2-4) requires a more aggressive approach and because these cases result in fibrosis of the preputial tissue surgical correction.

Prior to bandaging the prepuce the wounds should be cleaned and an attempt should be made to replace the prolapse. This can be facilitated with hydrotherapy (water hose spray, showerhead spray, or soaking). Soaking the prolapsed prepuce in a hypertonic solution (water with salt and/or sugar added) with the addition of povidone iodine also serves to aid in the debridement of the tissue. Then after drying an ointment is applied. The author prefers the petrolatum, tetracycline, and scarlet red oil mixture (Petercillin-Auburn)[5] for severely traumatized, necrotic wounds and utilizes less irritating ointments (Sugardine or mammary infusion ointments) for less affected tissue or after several days of treatment when the tissue is less inflamed. A 6-10 inch rigid plastic hose (milk line or equine nasogastric tubes can be used) is then placed within the prepuce to allow urination and the prepuce is wrapped with elastic tape (Elasticon™, Johnson and Johnson, New Brunswick, NJ). In addition to facilitating urination, the placement of the tube helps decrease the circumferential scarring down of the preputial orifice which results in phimosis. Bandage change intervals are dictated by the extent of damage, the bull's tolerance for the bandage, and whether or not you can completely replace the prepuce within the sheath. Following resolution of swelling (infection/inflammation) a decision can be made to return the bull to use or surgically correct.

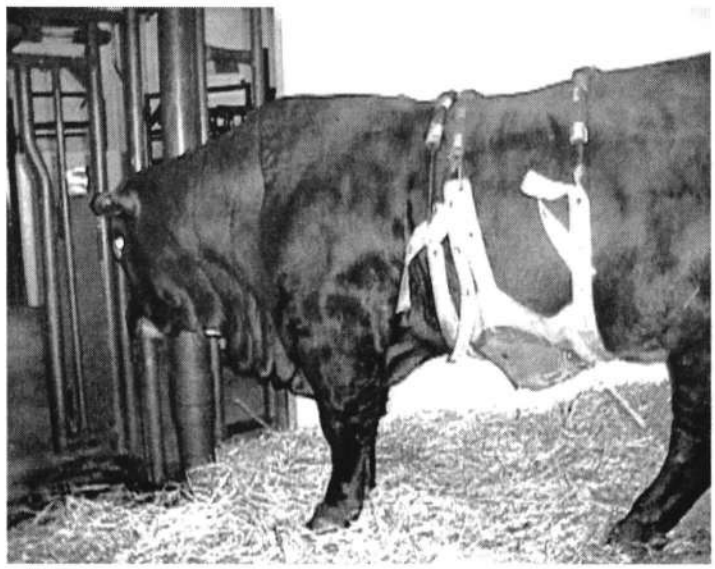
Often a client will choose to cull, rather than allow treatment. Because these bulls are the victims of severe price discrimination the author will offer a salvage type procedure. A large swine rectal prolapse ring or short length of polyvinyl chloride (PVC) pipe is placed within the preputial lumen dorsal to the lesion and a band is placed around the prepuce. This will amputate the damaged prepuce, but fibrosis and stenosis will likely occur. Alternatively holes can be drilled in the PVC pipe prior to placement and rather than a band, heavy (6 mm) synthetic suture material is utilized in an overlapping pattern that serves to both fix the tube and provide for hemostasis when the end of the prepuce is amputated. This is the "ring amputation" procedure and can be performed with the bull standing, utilizing local analgesia and chute restraint. This procedure usually results in some degree of stricture, and also may be considered for the client that desires a more economical option than treatment followed with surgical correction by circumcision. It should be noted that correction with the circumcision technique remains an option for bulls in which the ring amputation resulted in a stricture.

The surgical technique that is advocated by the authors is the circumcision or "reefing" technique. This procedure can be performed with regional analgesia, specifically the pudendal nerve block (described in: Management of urogenital injury and disease in the bull: the scrotum and its contents. Clinical Theriogenology, Volume 4, Number 3) and mild sedation. Prior to surgery, the bull is fasted for

48 hours and water withheld overnight. The bull is placed in right lateral recumbency and the hair of the sheath is clipped. The penis is extended and maintained in extension with towel forceps that engage the apical ligament. The penis and prepuce are prepared for surgery (no alcohol) and draped. Apply a tourniquet (one inch Penrose tubing) proximal to the area to be transected. The amount of prepuce to be resected is then determined, marker sutures (to insure that tissues are returned to the proper alignment) are placed, and two circumferential incisions are made. These incisions are joined with a longitudinal incision. These incisions are to be very superficial so that with careful, sharp dissection; underlying tissue, blood vessels, and lymphatics will be spared. The area of fibrosis should be included in the tissue removed. Following dissection and tourniquet removal (the tourniquet can be maintained safely for up to one hour); hemorrhage is controlled by vessel ligation and/or cautery. When hemostasis is achieved, the area is lavaged with a warm solution of sterile saline and povidone iodine solution (50 ml povidone iodine per liter of saline). The edges are sutured with a simple continuous subcuticular pattern using the surgeon's choice of 2-0 absorbable suture material.



Do not use one continuous suture, but instead end the pattern and re-start in three stages to avoid a constrictive (purse-string) effect. Do not close dead space. Follow this with a row of staples. Then suture in place Penrose tubing over the end of the penis. An antibiotic ointment is applied to the wound and placing the free end of the Penrose tubing into a 6 to 10 inch rigid tube, the penis and prepuce are carefully returned into the sheath and bandaged. The bandage can stay on as long as a week; the staples can be removed in two weeks. A support wrap (bull diaper) can be employed to protect the bandage and prevent pendulous swelling.



Preputial injuries that occur in bulls of English or Continental breeds typically result in phimosis rather than prolapse and therefore represent a different sort of challenge. If the penis is forcefully extended and then not repaired and replaced within the sheath at the same time, paraphimosis may result. The prepuce can be lavaged with an antiseptic solution. Then apply an ointment of the operator's choice and allow second intention healing to occur. Ascertain the extent of fibrosis and whether or not the penis can be extended (some bulls that can extend will still require surgery). The scar tissue must be removed to allow easy, painless, and full extension of the penis. Unlike the Brahman influenced bull, these bulls rarely have enough preputial tissue to allow for a circumcision. A scar revision technique is utilized. Following a pudendal block and preparation as described previously, an elliptical incision than includes the scar is made. Simply stated, the incision is made on whatever plane necessary to facilitate dissection of the scar, but closed on a longitudinal plane. Closure technique is crucial. A "bootlace" pattern is initiated distally (on the extended penis) and then continued proximally.

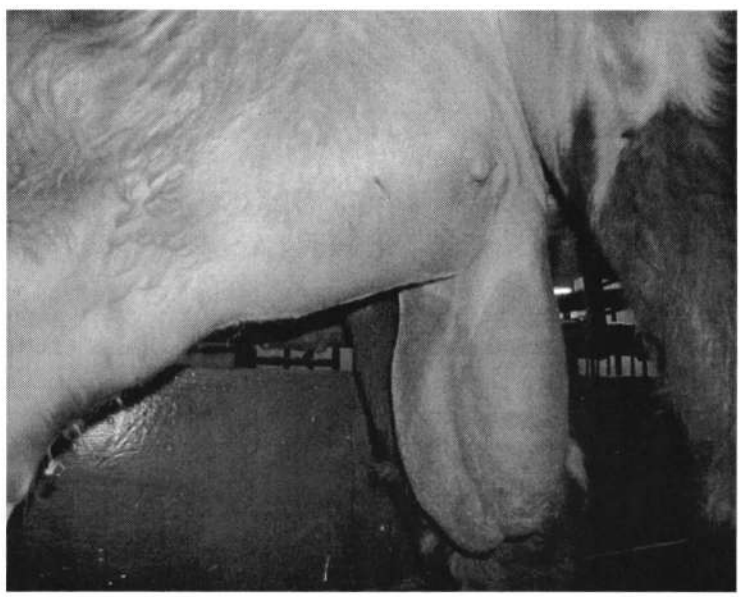
After completion the penis is released and allowed to return back within the prepuce before the suture ends are tied.[5] Tightening and tying off the suture prior to this will prevent retraction. A Penrose

drain is sutured to the end of the penis to facilitate urine drainage as described in the previous technique, but bandaging is usually not required. The sutures can be removed in two weeks or an absorbable suture can be used. The penis should be extended and examined prior to return to service.

In the case of either circumcision or scar revision the bulls should receive three months of sexual rest prior to return to service.

Hematoma of the penis

Hematoma of the penis is probably the most common injury of the penis. This injury occurs when the penis misses the intended target, hits the cow's perineum, and bends. Although it usually occurs in young inexperienced bulls[5-8] it can also occur in the older bull, which due to orthopedic injury or pain has altered his approach during mounting. The hematoma results due to rupture of the tunica albuginea and the subsequent hemorrhage from the corpus cavernosum. The initial volume of blood escaping is less than 250ml[5] but the size of the resultant hematoma varies as each subsequent erection results in further hemorrhage. Thus the hematoma may range in size from 15 to 30 cm. The resultant swelling occurs in the sheath over and cranial to the rudimentary teat.

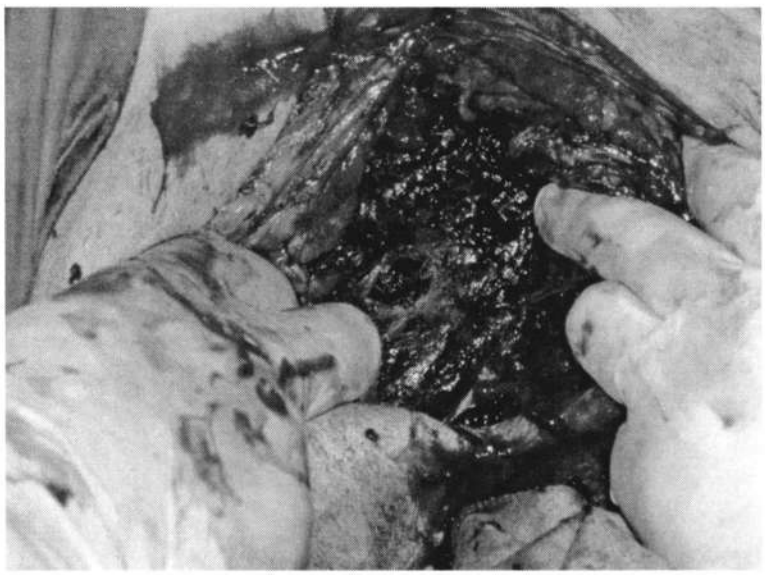


A prolapsed prepuce often results and this in fact may be the owner's reason for seeking help. Diagnosis may be aided with ultrasound. Do not attempt to aspirate the swelling as inadvertent introduction of bacteria can convert a hematoma to an abscess. From a prognostic standpoint, smaller (<20cm) hematomas respond equally well to conservative, medical therapy as they do to surgery, but larger (>20cm) hematomas have a much better response (75-80% versus 33%) with surgery.

Regardless which treatment plan is to be followed, systemic antibiotic treatment should be started. Medical treatment consists of continuation of antibiotics (procaine penicillin- 22,000 IU/kg, IM, bid), hydrotherapy, and sexual rest. Surgical correction should occur immediately. Given that the owner or attendant typically may not notice this problem for two to three days, that the patient must be fasted for two days prior to surgery, and that after 10 days organization of the clot and fibrosis has occurred; time is of the essence.

Once the decision is made to attempt surgical correction, antibiotic therapy is instituted if not already begun and feed is removed. The bull is fasted for 48 hours and water withheld overnight. General anesthesia can be utilized or regional anesthesia with heavy sedation. Either way the bull is tabled in right lateral recumbency with the left hind leg pulled back and up; fastened securely. The surgical area is prepared and a vertical incision 20-25 cm long is made just cranial to the rudimentary teat.

Careful dissection, attention to hemostasis, and manual removal of the blood clot follows. Lavage with a warm saline povidone iodine solution aids in the removal of additional clots and coupled with blunt dissection, identification of the lesion. Careful blunt or manual dissection is not just preferred over scalpel or scissor dissection, but mandatory as injury to area vasculature and nerves must be avoided.



Once the tear or rent is identified, carefully lavage the area again and debride the often tattered edges of the rent. This must be minimal as excessive removal of tissue complicates closure and following healing there is the potential that the bull will have trouble extending his penis. Closure of the defect with number. 1 polyglycolic acid (PGA) in a bootlace pattern is the long-standing recommendation and justifiably so, as there is enough wound tension to make simple interrupted suture patterns problematic. The elastic layers over the penis can be closed with 3-0 chromic catgut in a simple continuous pattern. The penis is then returned to its normal position, the subcutaneous tissues are closed with 0 chromic catgut and the skin can be closed with 6 mm synthetic non-absorbable suture material typically with a Ford interlocking pattern. Antibiotics are continued for five to seven days. The bull should have 60-90 days of sexual rest following surgery. Seroma formation occurs often enough that it probably should not be considered a true complication.

Complications include abcessation, suture dehiscence, reoccurrence in subsequent breeding seasons, and permanent analgesia[9] of the penis. Loss of sensation, however, likely results from the initial injury, rather than the surgery.

Penile deviations

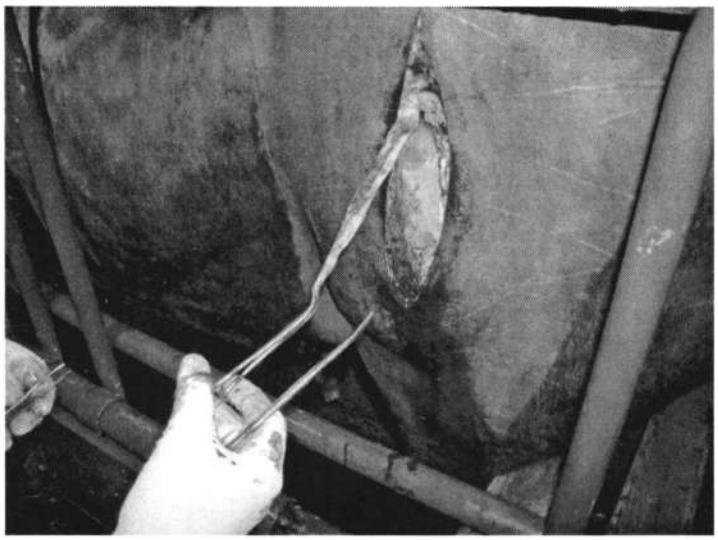
This discussion will be limited to diagnosis of the two common deviations; spiral deviation (corkscrew) and ventral deviation. Both likely result from abnormalities of the apical ligament and both are amenable to surgical correction.[10,11] Diagnosis of the spiral form should be made by observation of a natural mating attempt. The penis normally spirals to some extent upon intromission and this same level of spiraling is often manifested during electroejaculation. When this is severe enough to interfere with normal copulation the decision must then be made to cull the bull or attempt correction. Diagnosis of a ventral deviation can be made by observation during electroejaculation or mating.

Ventral deviation of the penis

Spiral deviation of the penis

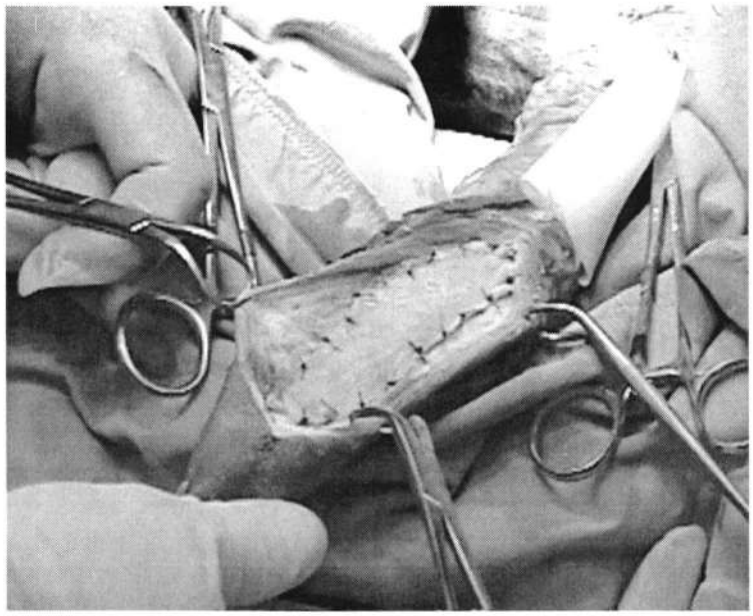
The surgical procedure which the authors recommend for correction is the fascia lata implant technique previously described by Walker and Young.[12] A rectangular strip of fascia is harvested from the bull, cleaned (areolar tissue removed), and placed between the apical ligament tunica albuginea. Alternatively, synthetic surgical mesh material can be used to substitute for the fascia implant.[13] Utilization of the mesh has the obvious advantage of removing the time-consuming fascia harvesting step and has recently been successfully utilized without complication. However, problems with post-operative infection with the mesh materials of the day along with dissatisfaction with the apical ligament "strip" technique were the impetus stated by Walker for the development of this technique.[12] Thus, the fascia lata technique will be described and those that prefer can easily modify the technique to utilize surgical mesh.

The bull is fasted 48 hours and water withheld overnight. Depending on his nature, the bull is sedated with 10-20 mg xylazine and 10 mg acepromazine IV. With the bull restrained and standing, the surgical site, an area on the upper left hind limb, is prepared since the bull will later be placed in lateral recumbency on his right side. A local block utilizing an inverted L injection pattern is administered. A 15-20 cm incision is then made utilizing the patella and greater trochanter as anatomical guides, with the incision being midway between. When the fascia lata is exposed, remove a rectangle shaped section. Removing a 3 cm wide by 15 cm long section will provide more than enough tissue for the graft.



Place the tissue in saline maintaining sterility and suture the edges of the fascia with a continuous pattern utilizing any number 1 or 2 absorbable suture. Failure to do so will result in painful muscle herniation. Close dead space and suture the skin with number 3 nonabsorbable synthetic suture material utilizing a Ford interlocking pattern. The harvested tissue is then prepared by rinsing in saline and removal of any attached tissue.

The bull can then be prepared for the placing of this graft material or synthetic mesh material of the same size. As with the other surgeries described, general anesthesia can be utilized or regional anesthesia with heavy sedation. Next the bull is tabled in right lateral recumbency and the preputial area is clipped and prepared. The penis is then extended and the apical ligament identified and grasped with towel forceps. The penis is prepared with povidone iodine surgical scrub, rinsed with sterile water or saline, and dried. Unlike when performing a circumcision a tourniquet is not utilized as the surgeon will want to be able to easily visualize the area vasculature. An incision is made on the central dorsal aspect of the penis from a point 3 cm from the tip and extending 20 cm proximally. With careful dissection, identify the apical ligament and incise through it for its entire length. This incision will expose the tunica albuginea and this is where the fascia or alternatively the synthetic mesh implant will be placed. The proximal aspect is placed first and sutures (2-0 chromic catgut) are placed in the corners attaching the implant material to the tunic. Interrupted sutures are then placed on the lateral sides of the implant, stretching the implant so as to avoid crumpling of the tissue. Care is taken to not penetrate too deeply into the tunic and to avoid suture placement that impinges on the dorsal vasculature. The distal end of the implant is trimmed if necessary and the distal end is sutured as previously described.



The edges of the apical ligament are then closed utilizing 0 chromic catgut with every other or every third suture engaging the implant. This suture will keep the apical ligament from slipping to the side which is crucial in correcting a spiral deviation. The elastic tissues and dead space are closed with a continuous suture pattern (3-0 or 4-0 chromic catgut) and the skin can be closed with 0 chromic catgut or other absorbable suture using an interrupted pattern. The incision site is gently rinsed with a dilute povidone iodine solution, an antibiotic ointment (bovine intramammary infusion medication) is liberally applied, and the penis is returned to the prepuce. Systemic antibiotics are administered for four to five days and the penis can be manually extended for examination in seven to 10 days. Three months of sexual rest is recommended.

Conclusion

Increasing sale prices for bulls should increase demand for these procedures. Foregoing general anesthesia through the use of regional analgesia, sedation, and proper restraint decreases the cost associated with these surgeries, making these procedures more economical for owners and more profitable for the veterinarian. Careful evaluation of penile and preputial injuries provides better diagnosis and allows for a more valid prognosis. All of these factors could possibly combine to signal a return to the time when bull doctors ruled the veterinary world!

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13. Warner G: Personal communication.

Addenda

Preparation of an autogenous wart vaccine

- Excise wart material
- Trim off exterior material and clean with soap and water
- Mince tissue with scissors or scalpel and mix with saline 10:1
  Or mix with saline and place in blender
- Strain homogenate through gauze
- Add 5 cc of 10% formalin to 95cc homogenate
- Incubate for 24 hours at 37° C
- Culture a small aliquot for 24 hours to check for sterility
- Balance pH with bicarbonate solution
- Refrigerate until used
- Inject subcutaneously

OWNER CONSENT and when dispensed the author provided epinephrine.

Material list for construction of a "bull diaper"

- 11/2 yards of heavy cotton material
- Large laundry bag for the netting
- Nylon straps- (3 per diaper)
- Grommets --hardware or craft store
- Bungee cords
- Pipe insulation for padding
- Duct tape

Petercillin recipe

- 500 g anhydrous lanolin
- 2 g tetracycline powder
- 60 ml scarlet oil

Editor's Note: The photographs in this paper appear in color in the online version of Clinical Theriogenology (http://st.omnibooksonline.com/).

Towards understanding early embryonic loss in mares: an experimental approach

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Introduction

Investigation of the clinically important problem of early embryonic loss in mares predates ultrasonography, predates transrectal palpation and is perhaps the earliest documented example, at least in the English language, of application of scientific thought and method to horse breeding. In a masterful 1897 monograph,[1] James Cossar Ewart (Figure 1) explains

that he undertook his study of "A critical period in the development of the horse" in response to a query from his patron, Lord Arthur Cecil, as to why so many mares, thought to be pregnant, return to estrus from the sixth to ninth weeks after breeding. What is of such enduring appeal in Ewart's approach to the problem is its comparative foundation; to arrive at his conclusions he builds an hypothesis by considering analogous developmental events in the chick, opossum and mare – an approach that is as valid today as it was then, despite the enormous changes in the criteria and methods used to make the comparisons.

Horses have been part of the human experience for more than 300 centuries,[2] domesticated for some 55 of those centuries,[3] but studied scientifically for little more than the most recent one. If it seems esoteric to read of early domestication when a more pressing problem is getting one's own mare in foal, and keeping her that way, it is mentioned to underline the fact that intervention is an essential component of horse breeding. It is intervention that has produced all the splendid diversity, beauty, and superb performance of our horses, true, but that same intervention has demanded that mares and stallions reproduce under less-than-natural circumstances. That may be asking a lot of them, which is why an old Lincolnshire adage has it that "if you want to lose a friend, buy him a brood mare; if you want to bankrupt him, buy him two".[4] The implication that horses are inherently difficult to breed is wrong: left to their own devices in the wild, in feral populations, or even free-ranging at pasture,[5] they are satisfyingly fertile. Asked to conceive and foal regularly when rarely meeting a stallion except at breeding time, unable to choose her mate, and sometimes urged to give birth as early in the year as possible, some mares are going to be less fertile. Perhaps we shouldn't be surprised.

However, it is domesticated, managed mares that we have to deal with and the extent of the problem of their early embryonic loss is considerable. This paper will therefore summarize the evidence for saying that the loss is indeed problematic, describe the experimental approach that we are taking to its investigation, discuss our findings in relation to the special case of twin reduction, and finally speculate on how field observations could help clarify some underlying causes of pregnancy failure.

Keywords: Early embryonic loss, luteolysis, twin and triplet pregnancy,

The extent of the problem

Defining when and to what extent pregnant mares lose their embryos obviously depends on first diagnosing pregnancy which, before the advent of ultrasonography, was performed by palpation. Transrectal examination of horses only really came into its own in the 1930s, and for very practical reasons. Abigail Woods[6] has described how, in Britain, the period between the two World Wars was one of transition for horse breeding; the era of working and military horses was over whereas "pleasure horses" and horse racing were as popular as ever. Thoroughbreds, in particular, were fetching high prices and so any reduction in their fertility was of economic concern, recognized as such by both the Thoroughbred Breeders' Association and the Horse Racing Levy Board. To their credit, these two bodies set about financing some of the very first research into equine "sterility" by veterinary surgeon F.T. Day at the Cambridge University Institute of Animal Pathology under the watchful eye of one of the "fathers" of reproductive biology in large animals, John Hammond. By using what was then rated as the "new" reproductive technique of rectal palpation, Fred Day was able to lay the foundations of modern knowledge of ovarian function and early pregnancy diagnosis, much as McKenzie and Andrews were doing on this side of the Atlantic, in Missouri, at that time. Strange as it may now seem, one precaution recommended for those practicing the technique was to have short fingernails. There is something satisfyingly rural about Fred Day's criteria for gauging the stage of pregnancy from the size of the conceptus in the uterus: a shell-less bantam's egg at Day 16, the eggs of the pheasant, hen, turkey, goose and ostrich at Days 25, 30, 40, 45 and 70, respectively. One is left wondering how familiar are today's graduates with these eggs! Nevertheless, it was clinical acumen and sensitive fingers that first revealed that the equine conceptus moves around in the uterus, the placement of the conceptus not always coinciding with the side of ovulation. Substantiation and elaboration of this fact was among the first contributions of ultrasonography to equine reproduction in the early 1980s. Ultrasonography, needless to say, has been key to defining when pregnancies are most likely to fail, thereby focusing research on those stages in an effort to reduce losses. There have been many reviews of the incidence and timing of early pregnancy loss (see[7] for references) but a particularly cogent study revealed that some 17% of pregnancies detected by ultrasonography at about Day 15 will not result in a foal, and most (60%) of those losses will be incurred before Day 35.[8]

Those figures – 17% and 60% – underlie our experimental approach to the study of early pregnancy failure; the 60% justifies a focus on the first month of pregnancy while the 17% indicates that waiting for naturally occurring cases would not be feasible logistically. Our solution has been to precipitate failures by inducing luteolysis in pregnant mares with the prostaglandin cloprostenol and then to compare the conceptuses and endometrial biopsies from these treated mares with those from saline-injected control mares.[9]

Materials and methods

Animals and experimental design

Thirty mares, of mixed breeding (mostly Standardbred and Thoroughbred) and unknown ages, were used under conditions approved by the University's Animal Care Committee. The herd numbered between 11 and 18 over the six years of the main study (2005 to 2010), with an overall conception rate (pregnancies per cycle with insemination for this and related studies) of 223/314 (71%). The mares were teased with a pony stallion, examined daily by ultrasonography when in estrus, and artificially inseminated with fresh semen from the pony stallion on alternate days until ovulation was detected (Day 0.5±0.5). Daily ultrasonography was resumed on Day 11.5 or 12.5 for pregnancy diagnosis and measurement of conceptus diameter and was continued until the day of conceptus recovery by transcervical lavage with phosphate-buffered saline.[10] Non-pregnant mares were 're-cycled' by intramuscular injection of 175 μg of cloprostenol in 0.7 mL (Estrumate, Schering Canada, Inc.) to induce their return to estrus.

The basic experimental plan to investigate the effects of luteolysis on the pregnancies obtained is depicted in Figure 2; the aim was to compose groups of five singleton pregnancies in

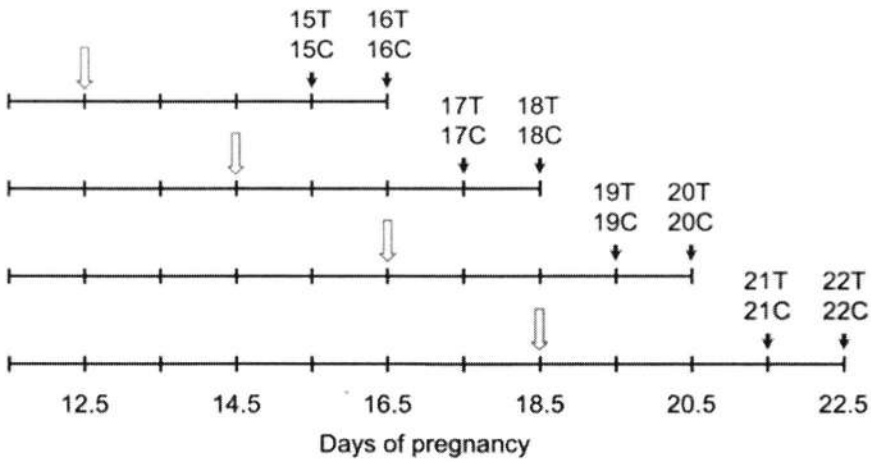

Figure 2: Use of pregnancies diagnosed at Day 11.5 to constitute the 8 experimental groups. On the days indicated, pregnant mares were injected (open arrows) with PG (treated, T) or saline (control, C) before conceptus recovery 3 or 4 days later (solid arrows).

both control and treated categories on each day between Days 15.5 ± 0.5 and 22.5 ± 0.5. For convenience, these groups are referred to as Day 15C etc. and 15T etc. for the control and treated categories respectively. Mares were allocated alternately to the treated or control category for their first pregnancy in a given year, then to the other category, alternating, for subsequent pregnancies in that year. On the day of treatment (Day 12, 14, 16 or 18), mares in the treatment groups were injected intramuscularly with 250 μg cloprostenol in 1.0 mL (PG); control mares received 1.0 mL saline (0.9%, w/v, NaCl) intramuscularly. The mares in each category were evenly allocated to groups scheduled for conceptus recovery either 3 or 4 days later. The two treatment–collection intervals were chosen in order to compare their putative effects and to increase flexibility in scheduling collections.

When the day-16 treatments were initiated, it was not known for how long the pregnancy would be maintained and yield tissues 3 or 4 days later. Therefore, to test the feasibility of using such a group, 4 mares were injected on Day 16 (3 treated and 1 control) and uterine flushes were scheduled for Days 21, 22 and 23, beyond the Days 19 and 20 eventually used in groups 19T, 19C, 20T and 20C.

Because of findings during the first 5 years (see Results), two further groups were added to the basic design for the sixth year: conceptuses from mares injected on Day 18 were collected either 1 or 2 days later, forming groups designated 18/19T, 18/19C, 18/20T and 18/20C.

Plasma from jugular blood samples, taken daily between the day that pregnancy was diagnosed and the day of conceptus collection, was stored frozen for progesterone assay.

Conceptuses that disappeared from the uterus (as judged by ultrasonography) between two successive examinations, leaving no trace in the subsequent uterine flush, were considered to have been aborted. Conceptuses recovered as shrivelled, blanched and/or fragmenting tissue were classified as degenerate. Remaining conceptuses recovered were deemed to have survived.

Over the 6 years, the mares were used repeatedly for a total of 163 conceptus recoveries (1-12 flushes per mare; mean and median 5.5 and 5 flushes per mare, respectively; excluding the 4 mares used to test pregnancy maintenance for >5 days after injection at Day 16). Twin and triplet pregnancies were not considered comparable with singletons and were not included in comparisons; conceptuses that ruptured completely or collapsed within an intact capsule were less than ideal for comparisons because of the loss or dilution of yolk-sac fluid. Consequently, unevenness in the distribution of pregnancies across

groups was inevitable as groups of 5 intact, single conceptuses per group were built up as far as was possible.

Measurement of yolk-sac expansion.

From the day that pregnancy was confirmed until the day of conceptus recovery, diameters of conceptuses were measured by ultrasonography. However, comparison of the growth rates of conceptuses of treated and control mares was limited to the period between Days 11 and 16 when the conceptus produces a circular or ellipsoid image on the screen, can be measured reliably and repeatably, and exhibits a linear increase in diameter.[11] The diameter of the conceptus was taken as the average of the horizontal and vertical diameters of its image on the screen when 'frozen' at its maximum size.

Sample collections

Conceptuses were washed in ~400 mL fresh PBS before transfer to a Petri dish for photography and dissection. In the case of intact conceptuses, yolk-sac fluid was collected after puncture, centrifuged to remove debris, and stored frozen pending analyses.[12] Dissection of all conceptuses was completed under phosphate-buffered saline (PBS). First, the capsule was removed and stored frozen in 0.5 mL PBS; if detached from the yolk sac, the 'completeness' of the capsule (the likelihood that it had enveloped the whole yolk sac) was estimated. Then the embryonic disc was examined and photographed to record somite development before removing it, or the embryo proper, as well as the bilaminar and trilaminar regions of the yolk-sac wall for related genomic and proteomic studies.[13-16] At Day 19 and later the integrity of the blood circulatory system was assessed by examining embryos proper for heartbeats, and vitelline vessels for the presence and movement of erythrocytes.

Yolk-sac fluid measurements

Fluids recovered from conceptuses from treated and control mares were compared with respect to their osmolalities and concentrations of progesterone, estrone sulphate and protein.[9]

Observations on twin and triplet pregnancies

Conceptuses from 31 twin- and two triplet-pregnancies, not included in the comparisons of conceptuses from treated and control mares because of their propensity to be lost during the first month of pregnancy, have been collected during these experiments and studied morphologically to help clarify how twin reduction can occur naturally.[17]

Statistical analyses

Conceptus recovery rates were compared by Fisher's exact test. Osmolalities, protein and steroid concentrations in yolk-sac fluids were analyzed by analysis of variance, somite counts by Student's *t*-test (GraphPad InStat version 3.00 for Windows 95, San Diego, California, USA).

Results

The luteolysis experiment

Luteolysis was induced in all the PG-treated mares but in none of the controls.

From the 123 singleton pregnancies in which recoveries were attempted between Days 15 and 22, about half of the conceptuses were recovered intact, the rate dropping from 67% (37/55) overall for Days 15–17 inclusive to 40% (27/68) overall for Days 18–22 (P=0.004).

The patterns of increase in diameters of conceptuses measured *in utero* up to Day 16 were not significantly different in untreated (i.e. before treatment), treated and control groups and were linear ($y = 3.81x - 36.89$; $r^2 = 0.995$). Furthermore, the volumes of yolk-sac fluid recovered from intact conceptuses *in vitro* were similar in the treated and control groups.

Conceptuses recovered from mares in treated and control groups on either Day 15 (9 treated, 8 controls) or 16 (4 treated, 4 controls), were indistinguishable on their respective days. Up to, and including, Day 18, all intact conceptuses from the 24 treated and 20 control mares were completely

enveloped in capsule. Subsequently, some yolk sacs were recovered intact but with a separated capsule. Envelopment by the capsule was complete in 4/8 intact conceptuses on Day 19, 3/8 on Day 20, and 1/5 on Day 21. It was possible to supplement these data with estimates of the completeness of a further 38 capsules that were found detached from both intact and ruptured yolk sacs collected over the same stages of pregnancy. Overall estimates of the proportions of complete capsules then became 6/11, 3/7, 1/6 and 0/5 on Days 19, 20, 21 and 22, respectively, for conceptuses from control mares, and 7/9, 4/7 and 1/1, respectively for the first three of those days for conceptuses from PG-treated mares.

In conceptuses recovered with intact capsules, the nature of the capsules differed between those from treated and control mares. On Days 17 and 18 the capsule remained resilient and smoothly spherical in 10 of 11 intact conceptuses recovered from PG-treated mares but in only 2 of 7 conceptuses from control mares (P=0.013). In the remainder of the conceptuses the capsule was flaccid and pendulous. Similarly, in groups 19T and 20T, 6/7 capsules from treated mares remained closely applied to the yolk sac versus 0/3 capsules in the control group, in which the capsules were more pendulous (P=0.033).

With considerable variation within days, the neural plate, neural tube and somites developed similarly in singleton conceptuses recovered from treated and control mares. Blood appeared near the neural plate on Day 18 in both groups. Numbers of somite pairs in treated and control groups did not differ significantly and, overall, the increase with developmental stage was linear. Evidence of a heartbeat and/or circulating blood was found fairly consistently by Day 19 (4/5 in group 19T, 2/5 in 18/19T; 6/6 in group 19C, 4/5 in 18/19C). Such evidence of a functioning circulatory system was not regularly sought when dissecting younger conceptuses but was nevertheless recorded in 4/12 conceptuses in group 18C.

Buoyancy of intact conceptuses in the PBS flush fluid up to and including Day 18 was complete (i.e. to the surface) in 23/24 from treated mares and all 20 from control mares. On and after Day 19, most intact conceptuses sank: 10/11 from treated mares, 8/9 from control mares. Accordingly, the osmolalities of the yolk-sac fluid of conceptuses from treated and control mares did not differ statistically; for the combined data, analysis of variance indicated that the increase in osmolality with time was significant.

Concentrations of protein, estrone sulphate and progesterone in the yolk-sac fluids were unaffected by PG treatment. Analysis of variance indicated significant increases in concentrations of protein and progesterone, but not of estrone sulphate, with time.

To summarize these morphological and endocrinological results[8], when luteolysis was initiated on or before Day 16, most pregnancies survived until the time of collection and the conceptuses in respective treated and control groups on Days 15 to 20 were very similar except for some effects of treatment on the capsule and vascular development. In contrast, after luteolysis was initiated on Day 18, abortion often ensued within 3 days and most conceptuses collected had degenerated (Figure 3), therein constituting a predictable system in which to study the pathogenesis of a particular cause of pregnancy failure.

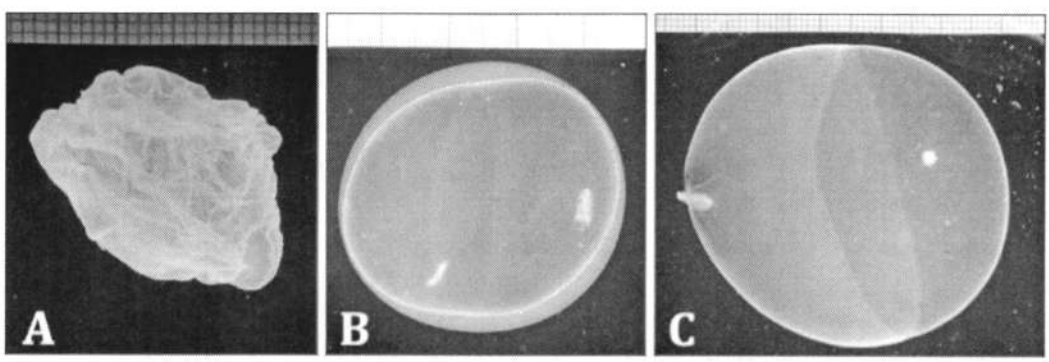

Figure 3: Day-21.5 conceptuses from PG-treated (A,B) and control (C) mares, with the embryo proper at 8, 3 and 9 o'clock, respectively. Note the complete lack of vascularity in A and B and the total collapse in A. The scale (top of each image) is in mm.

We are now examining when and how day-18 PG first exerts its adverse effects by comparing conceptuses collected at 24, 36 and 48 h after injection of the mare.

We have also compared treated and control pregnancies using proteomic, gene expression and immunohistochemical studies. Some of the changes in the more abundant proteins such as β_2-microglobulin (β_2M), secretory phospholipase A2 (sPLA2) and uteroglobins were revealed by gel electrophoresis and amino acid sequences [12-14]. More recently, we have compared proteins present in smaller amounts by direct mass spectrometric sequencing of trypsinized protein extracts of capsule, uterine secretions and endometrium [15]. In 2010, we started to compare transcriptomes of endometrium and trophoblast using the Agilent 60-mer 44K Horse Expression microarray. These experiments extend over 3 breeding seasons (2009-11) to provide sufficient samples at various time-points. Preliminary results from 2010 demonstrated 11 trophoblast-expressed genes, including two cytokines resembling CXCL7, that were significantly (>2 fold) elevated 24 h after day-18 PG. Many more differences were observed in the endometrium. These data will be combined with 2011 samples to confirm major differences observed, verified by qPCR, and fully analyzed in 2012.

Our proteomic studies further indicate that the equine endometrium produces a repertoire of proteins for transport of hydrophobic molecules through the capsule, especially GM2 activator protein (GM2AP) and other lipid carriers including P19/uterocalin and uteroglobin. In compromised pregnancies, some endometrial glands have increased amounts of some proteins of unknown functions. After PG, bound sPLA2 increases along with its endometrial expression whereas FGF-binding protein (FGFBP) and connective tissue growth factor (CTGF) decrease.[16] These findings suggest that the capsule might be a reservoir of growth factors that act on the endometrium when and where they are released as the capsule is degraded during the third week of pregnancy.

Observations on twins and triplets

In the 31 twin- and two triplet pregnancies, signs of contact between conceptuses were deduced from those seen in one pair that remained attached by their capsules on Day 18.[17] Signs were mild in twins recovered before or during fixation; they were found on capsules in two of 10 pairs at Days 16-17 even though contact had not been seen by ultrasound. After fixation, the signs became stronger in seven of nine unilateral pregnancies, indicated adhesion between pairs, and included effects on the vitelline circulation and/or degeneration of one twin (Figure 4).

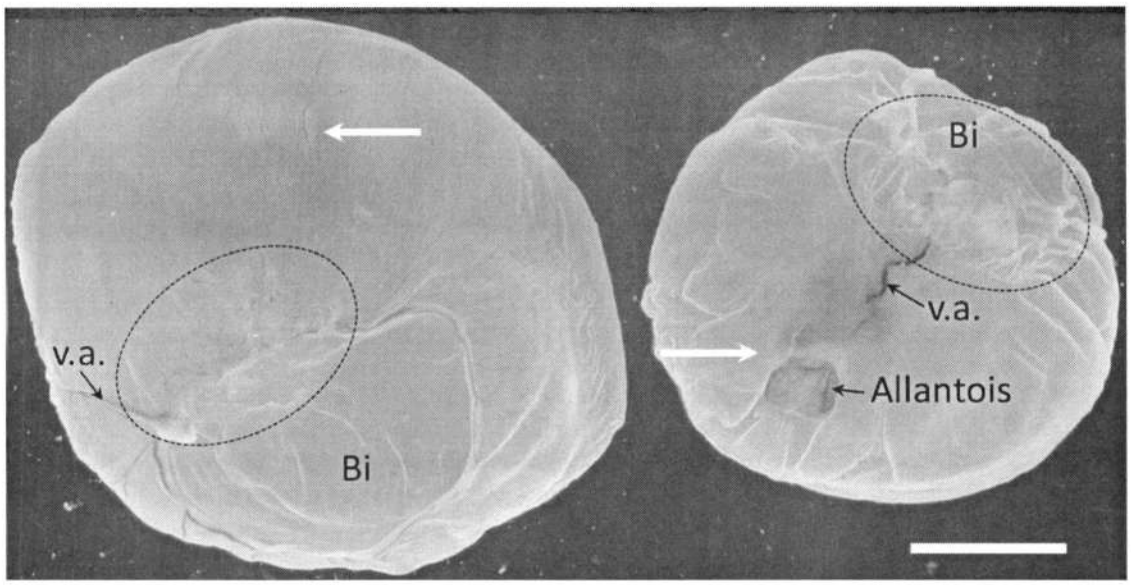

Figure 4: Twin conceptuses floating in PBS after recovery 24.5 days after two same-day ovulations. Each embryo proper (white arrows) had developed an allantois. However, blood is obvious in the sinus terminalis (encircling the regressing bilaminar yolk-sac wall, Bi) of the larger twin but conspicuously absent from the sinus terminalis in the smaller one. Identical ellipses have been drawn over presumed attachment areas. The vitelline artery (v.a.) in the smaller twin appears to be dilated and its patency terminates in the attachment area. A tag of capsule was found adjacent to this area. Scale bar indicates 1 cm.

Conceptuses recovered from five of seven unilateral twin pregnancies after the time of capsule disruption (~ Day 21) evidenced embryo reduction; in the two surviving pairs attachment between twins was near the trilaminar/bilaminar yolk-sac wall border, consistent with the notion that survival depends upon an unencumbered trilaminar yolk-sac wall.

Serendipitously, presumed contact between the twins in two day-16 pregnancies left haloes of material that marked the normally transparent capsule. Tracing the position of the marks, relative to the embryonic disc within, for 40 to 60 minutes showed that the yolk sac can move within the capsule.

Discussion

The luteolysis experiments

Unlike previous investigations of how prostaglandin-induced luteolysis affects early pregnancy in horses, our study[8] has characterized the effects of such treatment on the conceptus itself. The experimental design, besides providing embryonic and maternal tissues for concurrent genomic and proteomic investigation of early equine pregnancy,[13-16] allowed us to show that the effects of treatment change as gestation advances. Indeed, the most salient finding of this study is that the effects of prostaglandin are morphologically minimal within 3 or 4 days when the mare is treated between Days 12 and 16 (i.e. up to about the time of fixation) but marked after treatment at Day 18 (after fixation). Treatment during the earlier period does affect the capsule but the progress of normal expansion and development of its enclosed yolk sac and embryo, despite baseline concentrations of progesterone in the maternal circulation, is remarkably 'autonomous' until, at Days 19 and 20, adverse effects on the embryonic vascular system begin to show. In contrast, luteolysis initiated by prostaglandin injected on Day 18 was associated with direct morphological effects on the 'vulnerable' conceptus beginning within 48 h, primarily a collapse of the yolk sac accompanied by cessation of blood circulation in the embryonic vascular system. The reasons for the switch from autonomy to vulnerability are unknown but may include the onset of vasculogenesis at Day 18 and perhaps changes in water transport through the yolk-sac wall,[12,18-20] reflected in the change in conceptus buoyancy observed at Day 19.

We are identifying morphological, gene expression, protein and steroid profiles that differ in normal pregnancies and in pregnancies destined to fail as a result of day-18 prostaglandin treatment,

paying special attention to vasculogenesis and water transport. Interpretation of differences will open the way to identifying signals of pregnancy loss and suggest targets for its therapeutic prevention or avoidance. We appreciate that this model of pregnancy failure does not exactly mimic events that occur naturally. Rather, it provides a predictable means of identifying points of the conceptus–mare dialogue that are vulnerable to disruption, are potentially important to pregnancy failure from a variety of causes, and might be amenable to therapeutic correction. Thus, identifying key differences in, for example, uteroglobins and sPLA2 in the normal and compromised pregnancies, will facilitate the transition towards diagnostic use of our results by investigating methods for sampling the uterine environment transcervically, and also comparing flush fluids and endometrial biopsy samples from mares that have recently lost an early pregnancy.

Embryo reduction in twin pregnancies

Our direct morphological examination of the conceptuses from twin and triplet pregnancies have added to our knowledge of how the conceptuses in such pregnancies establish contact and adhesion to each other during the first month of pregnancy, the role of the capsule in that process, and how the adhesion can lead to the demise of one of the pair.[17] The findings indicate that contact and/or adhesion between the capsules of twin conceptuses is instrumental in bringing about longer-term adhesion, and that the site of contact and/or adhesion is important in determining whether both twins will survive. This has a bearing on alternative views about how twin reduction is normally achieved. According to the deprivation hypothesis proposed by Ginther, reduction of one member of a twin set occurs when a major portion of its trilaminar yolk-sac wall is in apposition with the other conceptus, rather than with the endometrium.[21] Conversely, survival of both twins is most likely when both have a minimum of their trilaminar yolk-sac walls apposed to each other. The latter situation can occur when orientation of the two conceptuses is symmetrical, such that one embryo proper lies in the four o'clock position and the other in the eight o'clock position on ultrasound images taken after the embryos proper become visible. However, developmental changes in the relative importance of nutrient uptake through the bilaminar and trilaminar parts of the yolk-sac wall remain poorly understood. Thus, the alternative view – that it is compromise of absorption through the bilaminar wall that brings about twin reduction[22] – cannot be excluded without further research, preferably experimental and in mares with a known propensity for twin ovulations. Just one twin pair in the present series argues against such a critical role for the bilaminar wall; attachment of the degenerate twin recovered from one mare at Day 21 had been confined to the bilaminar wall of the surviving twin without impeding its development.

Interference with the vasculature of the vitelline circulation (exemplified in Figure 4) seems a likely cause of the loss of a twin on some occasions.

These observations are not of clinical application now that the management of twin pregnancies by 'pinching' one conceptus is routine. They do, however, help explain how the presence of twin conceptuses in a single uterine horn is usually inimical to their survival. Our findings point to a role for the capsule in initiating adhesion between twins and thus participating in 'embryo reduction' which, in the absence of clinical intervention, is presumably of selective value in reducing the fecundity of twin ovulating mares. We have shown, too, that at least limited movement of the yolk sac within the capsule is possible and needs to be taken into account in considering the processes that are essential to the maintenance of pregnancy in mares.

Back to Lord Arthur Cecil's question

So why do so many mares lose their pregnancy within a few weeks of breeding? The short answer is that we still don't know. A longer, more informative, and more optimistic answer is that current proteomic and genomic tools, used in well-structured experiments, are rapidly providing probable explanations. Our approach of using these tools to study events during normal and failing pregnancies complements the ways in which others are using analogous methods to compare the uterine environments in pregnant and non-pregnant mares.[23-28] Collectively, equine reproductive research is entering a phase of

analyzing and interpreting enormous amounts of information and the fruit of such 'high tech' work will be exciting and useful.

It is also possible that distinctly 'low tech' investigations of horse behavior and management could help answer Lord Cecil's question. A recent investigation based on breeders' responses to a questionnaire in the Czech Republic compares the incidence of fetal loss in mares bred to a 'home' stallion with that seen in mares sent away to another stud for breeding and then brought home.[29] Over a period of 15 years, none of the 36 mares mated to a home stallion lost a pregnancy whereas 31% (14 of 45) mares sent away to a 'foreign' stallion did so. Furthermore, in the returning mares, loss was seven times more likely if they were separated from an adjacent home stallion (or dominant gelding) by a fence than if the mares were in the same enclosure as the male, and could copulate with him. The authors interpret their results

> " ... as suggesting that where possible, a mare manipulates the male's paternity assessment by promiscuous mating. If she has no chance to do that, she may abort the current foetus. We cannot explain the mechanism yet but speculate that this may be a mechanism actually activated by the mother".

Two facts and a supposition underlie this interpretation. The first fact is that, in some mammalian species, males that have mated with a given female are unlikely to kill their own offspring neonatally. Conversely, males are prone to practice infanticide on the progeny of other males in order to hasten their opportunity to sire their own young. The second fact is that females of over 100 mammalian species are known to mate with multiple males, perhaps to "confuse parentage", thereby avoiding infanticide and the consequent wastage of their maternal investment. The supposition is that frustration of repeated mating (i.e. by the males across the fence) leads to termination of the pregnancy "...as an adaptation to save energy and avoid likely future infanticidal loss of their progeny".

The authors argue against such pregnancy losses in mares being caused by the Bruce effect, which in rodents and wild gelada monkeys[30] causes females to abort in response to the introduction of a strange male.

These intriguing observations deserve experimental confirmation and extension. Who better to do so than equine clinical theriogenologists and their clients? Who would be more satisfied than James Cossar Ewart were such comparative and interdisciplinary studies found to have a bearing on the problem of early pregnancy loss in horses?

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The portrait of James Cossar Ewart is from the Department of Special Collections, Edinburgh University Library. The steel engraved portrait of Lord Arthur Cecil is from Baily's Magazine of Sports and Pastimes, 1902;77:177-179.

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

Clinical use of B-mode and color-Doppler ultrasonography to evaluate preovulatory follicle status in mares

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Abstract

This review will focus on the main findings of studies that used B-mode and color-Doppler ultrasonography during the preovulatory period to evaluate the morphological and blood flow/perfusion changes of the preovulatory follicle in mares. The topics to be addressed herein will be: B-mode ultrasonographic characteristics of the preovulatory follicle; blood flow and perfusion changes of the follicle wall; signs of impending ovulation; prediction of impending ovulation; types of preovulatory follicle outcomes such as ovulation, septated evacuation, hemorrhagic anovulatory follicle, atresia; vascularity of the preovulatory follicle versus fertility; and relationships between preovulatory follicle and corpus luteum blood flow in mares.

Keywords: Preovulatory follicle, blood flow, ultrasonography, ovulation, equine.

Introduction

Recently, transrectal Doppler ultrasonography has been utilized increasingly for research and clinical studies of ovarian and follicle hemodynamics in large farm animals. High-resolution ultrasonographic machines with B-mode (gray-scale) and color-, power-, and spectral-Doppler modes have brought a powerful dimension to the evaluation of the equine preovulatory follicle during recent years. These technologies have permitted the development of more in-depth scientific and clinical studies with regard to the characteristics of the preovulatory follicle and the ovulation process. Results of recent studies have demonstrated the potential of Doppler ultrasonography for providing clinical information on the status and future success of a follicle to ovulate and its oocyte to become fertilized and to generate an embryo/pregnancy. The equine model allows hypotheses testing using the three Doppler technologies for examining the ovaries and may provide additional information that can also be considered useful for other farm animal species and in human clinical medicine.

The use of color-Doppler technology to evaluate the vascularity of the follicle wall in mares started in 2004. Follicle blood-flow assessment by Doppler ultrasonography has been used in mares to study: (a) follicle selection, (b) anovulation during transitional seasons, (c) first versus later ovulations of the year, (d) follicle maturity and proximity to ovulation, (e) oocyte recovery rate, maturity, and quality, (f) normal versus abnormal ovulation (septated evacuation) or anovulation (hemorrhagic anovulatory follicles [HAFs] or luteinized unruptured follicles [LUFs]), (g) the relationship of systemic human chorionic gonadotropin (hCG) antibodies on follicle vascularity, maturity, and oocyte qualities, (h) age-related effects, (i) the relationship between preovulatory follicle and corpus luteum (CL) blood flow, and (j) potential for pregnancy establishment. Greater vascularity of the preovulatory follicle has been associated with greater follicle diameter (women, mares, heifers), retrieval rate of oocytes (women, mares, heifers), retrieval rate of mature oocytes (mares), in vitro fertilization rate (women, heifers), pregnancy rate (women, mares, heifers), and a lower incidence of triploidy (women). In addition, follicles with greater blood flow have resulted in better embryos and more pregnancies after embryo transfer in women.

This review article will summarize the main findings of experiments that used B-mode and color-Doppler ultrasonography during the preovulatory period to study the morphological and blood flow/perfusion changes of the preovulatory follicle in mares. Portions of this article have been adapted from Gastal 2011[1,2] and Gastal and Gastal 2011.[3] This review is directed to equine theriogenologists and scientists who are involved in monitoring, managing, and manipulating ovarian function in mares.

B-mode ultrasonographic characteristics of the preovulatory follicle

Follicle wall echotexture and thickness

Ultrasonographically detectable changes in gray-scale echotexture and color-Doppler signals of blood flow can be noticed in the wall of the future dominant follicle as early as one or two days before the beginning of follicle diameter deviation or selection in mares.[4,5] Thereafter, as the follicle matures and ovulation approaches, several ultrasonographic changes can be seen in the wall of the preovulatory follicle.

In regard to the follicular wall, the granulosa layer is identifiable as an echoic band enclosing the antrum.[6] The two layers of theca can be assumed on the basis of position, but the boundary between the theca externa and interna is indistinct. In the initial equine B-mode ultrasonographic study,[7] granulosa thickness increased as the interval to ovulation decreased. In another study,[8] mean scores of echogenicity and thickness of the granulosa increased during the 24 hours before ovulation, and changes occurred in 60% and 70% of individuals, respectively. In addition to an increase in echogenicity of the granulosa, prominence of an anechoic band in the expected area of the theca layers increased daily and progressively over three days before ovulation.[6,9] Follicle diameter and the two echotexture characteristics were more prominent earlier than later in the season.[6] Early in the season, both characteristics were at the maximal score in 70% (33/47) of follicles on Day -1 (ovulation = Day 0). The anechoic band is located in the area of the thecal layers and its characteristics have been described.[4,6] A similar anechoic band has been described in women.[10,11] Color-Doppler signals of blood flow are dispersed within the anechoic band, indicating that the band includes the fluid of blood vessels, presumably venules as well as arterioles. In another study,[12] several mean pixel values increased in an approximately linear fashion during the 14 hours before ovulation in hCG-treated mares. In a more recent study[13], mares with and without hCG treatment have been compared. An increase in granulosa thickness and echogenicity, percentage of follicle wall with color-flow signals and prominence of the signals, and a decrease in circulating estradiol during the 36 hours post-treatment were greater in the hCG group than in the controls. During 36 to 12 hours before ovulation, the granulosa thickness and echogenicity, and percentage of follicle wall with color-flow signals and prominence of the signals increased in both groups. However, four hours before ovulation, the two groups showed similar decreases in prominence and percentage of wall with an anechoic band and prominence and percentage of wall with color-flow signals. This study indicated that the ultrasonographic changes of the wall of the preovulatory follicle were not associated temporally with changes in estradiol concentrations and prominence of an anechoic band, and color-Doppler signals decreased during the last few hours before ovulation. In regard to age-related effects on preovulatory follicle echotexture and vascularity, a recent study has not found differences among young, intermediate, and old mares for granulosa thickness and echogenicity, anechoic band prominence, and blood flow during 4 days before ovulation.[14]

Follicle shape and turgidity

Changes in follicle shape from spherical to nonspherical can be noticed from three days before ovulation, with the highest frequency occurring within 24 to 12 hours before ovulation (reviewed in Gastal et al. 1998[6]). In a recent study, loss of spherical shape was associated to decreased follicle turgidity, indicated during transducer pressure, and occurred mostly between the last 24 to 12 hours before ovulation.[15] The subjectivity aspect for evaluation of the previous characteristics can produce different results between operators that can be at least partly attributable to the criteria used to differentiate between spherical and nonspherical follicles and variations with transducer pressure.

Indicators associated with impending ovulation

The above studies indicate that quantitative ultrasonic characteristics of the granulosa and anechoic band are useful for sequential assessment of the developmental progress of the preovulatory follicle. However, assessing progress requires judgment on relative changes either subjectively or by computerized pixel analyses. Discrete (nonquantitative) B-mode characteristics have been described for the preovulatory follicle and may be useful for predicting time of ovulation without the necessity for

judging quantitative progress. These characteristics include the following: 1) decreased turgidity of the follicle under transducer pressure in mares[16] and women,[17] 2) loss of spherical shape in mares[7,16,18] and women,[17,19,20] 3) irregular inner surface of the granulosa termed crenation in women[10,20] and serration in mares[15,21] (see below for details), 4) stigma, thin apical cone-shaped, or nipple-like protrusion of the follicle (future ovulation site) in mares[7,16] and women,[17] 5) apparent detached segments of granulosa or rent in the wall in mares[16,22] and women,[23] and 6) echoic spots floating in the antrum in mares[16] and women.[24,25]

Recently, we observed that both surfaces of the granulosa (interfaces with the antrum and with the theca interna) became irregular or with a notched appearance as ovulation approached and termed the phenomenon "serration of granulosa".[15] In this study, the following discrete end points were recorded as present or absent: 1) serration of granulosa, 2) decreased turgidity, 3) loss of spherical shape, 4) apical area, and 5) echoic spots floating in the antrum. When records were examined for 24 and 12 hour intervals, serration was detected at the last examination before ovulation in 37% and 59%, respectively. Decreased turgidity at the last 12-hour examination was detected concomitantly with serration, but was detected alone in 9 to 12% of previous examinations. Serration and decreased turgidity were present at each examination until ovulation five hours later. Loss of spherical shape initially occurred less frequently than decreased turgidity, but the incidence increased from 50 to 100% during six to one hours before ovulation. The incidence of an apical area reached 100% and echoic spots increased to 50% during one hour before ovulation. The results indicated that serration of granulosa and the other discrete characteristics were useful for predicting the time of ovulation within hours, but optimal efficiency would require examinations every few hours. In this regard, owing to the appearance of serration within the last 12 hours before ovulation and a decrease in follicle blood flow during the last hours before ovulation (e.g., six to four hours)[13,21] we have proposed a model for follicle maturity and impending ovulation in the mare. The working hypothesis was that during a few hours before follicle evacuation, the blood flow becomes concentrated to the base of the follicle and vessels protrude throughout the wall, causing the appearance of serration in the follicular wall opposite to the future site of follicle rupture. Although the presence or absence of an indicator of impending ovulation required judgment, the presence of discrete indicators would likely be more readily evaluated than the progression in thickness and echogenicity of the granulosa and prominence of an anechoic band described in other studies. Serration of granulosa was the most useful indicator of impending ovulation, considering its distinctive appearance and consistent development only during the late preovulatory period. Future work is needed to develop a system that utilizes serration for predicting the interval to ovulation in mares.

Follicle diameter

Repeatability of preovulatory follicle diameter. Repeatability of follicle diameters has been recently studied in mares.[26,27] Significant correlations were found for the diameter of the preovulatory follicle during the three days before ovulation in spontaneous waves[27] and for the maximal diameter of the preovulatory follicle in induced waves.[26] When induced waves with only one ovulation were considered, there were stronger and significant correlations for diameter of the preovulatory follicle at maximum ($r = + 0.70$) and on the day before ovulation ($r = + 0.66$). In another study,[28] most mares (85%) ovulated from follicles that were within 3 mm of the diameter during the previous estrous cycle.

The finding that the preovulatory follicle tends to reach a diameter that is characteristic of the mare may be a useful knowledge in equine breeding programs. The significant positive correlations for diameter of the preovulatory follicle recently found occurred during the three days before ovulation. These days encompass the day that the follicle first reaches ≥35 mm in approximately 70% of the interovulatory intervals (IOIs), and ≥35 mm is a common diameter for administration of an ovulation-inducing drug. These observations suggest that knowledge of the mare's history on the diameter preceding ovulation may be useful for estimating the optimal follicle diameter for a given mare for ovulation induction, as well as for the optimal time for breeding before spontaneous ovulation.

Factors that affect preovulatory follicle diameter. Preovulatory follicles in horses and large ponies generally reach 40-45 mm the day before ovulation. The preovulatory follicle was larger in

Quarter Horses (43 mm) than in Arabians (40 mm),[29] larger in one type of Thoroughbred than in another (44 vs. 41 mm),[30] and 3.1 mm larger for Thoroughbreds in Australia than in England.[31] The diameter or growth rate of the ovulatory follicle before ovulation was similar between ponies and Quarter horses.[32] However, the maximum diameter of the preovulatory follicle was approximately 3 mm larger in French saddle horses than in Welsh ponies.[33] Limited data suggested that preovulatory follicles were about 5 mm smaller in miniature mares and 10 mm larger in Clydesdales than in Quarter horses and large ponies.[34]

In a recent study, follicular dynamics was considered in miniature mares.[35] The mean diameter of the ovulatory follicle at maximum (37.3 ± 0.5 mm) was greater than on Day -1 (35.9 ± 0.6 mm). A reduction in growth rate of the ovulatory follicle between maximum diameter (1 or 2 days before ovulation) and ovulation was seen in the miniature ponies and is consistent with that reported for large ponies[13] and horses.[36-38] Knowledge on the diameter of the preovulatory follicle in miniature mares is useful in comparison of breeds and types, considering the extremely small body size.

In the same study[35,39] described above, a preliminary comparison was made among miniature ponies, large ponies, and Breton horses. The miniature ponies and the large ponies had a longer IOI than the Breton horses, agreeing with previous comparisons between large ponies and horses (reviewed in Ginther 1992[40]). The miniature ponies had fewer growing follicles ≥10 mm per ovulatory wave and more ovulatory waves with only one growing follicle ≥10 mm than for large ponies and horses. The diameter of the ovulatory follicle was smaller at maximum and on Day -1 in the Miniature ponies than in the horses, but was not different from the diameter in large ponies. The growth rate (approximately 3 mm/day) of the preovulatory follicle, prior to the cessation or reduction in growth, was similar among the three types of mares and agrees with reported studies in ponies and horses (reviewed in Ginther 1995[34]). Therefore, differences in diameter of the preovulatory follicle among breeds and types of mares have to be considered and incorporated in the reproductive management. These findings demonstrate that when body size difference is large (e.g., Miniature and draft horses), follicle size difference might be more pronounced among breeds and types of mares. However, these differences are small when contrasted to the great difference in body size.

Mares are seasonally ovulatory with the transition between the anovulatory and ovulatory seasons occurring in the spring. The diameter of the preovulatory follicle on the day before ovulation was greater before the first than before the second ovulation of the year.[41-43]

The effects of inadequate nutrition or poor body condition on follicle dynamics and reproductive hormones during the equine ovulatory season are not fully understood. Although, there is evidence that inadequate nutrition or body condition has been associated with delayed onset of the breeding season, decreased pregnancy rate, increased embryo loss, and increased gestation length in mares.[44-46] During the winter, mares with low body condition had fewer medium (11 to 19 mm) and large (≥20 mm) follicles than mares with high body condition.[47] The mechanisms by which feed restriction and low body condition modify the reproductive axis are also not well known. Apparently, glucose, insulin, leptin, growth hormone, and fatty acids seem to be involved, at some level, in the regulation of the reproductive axis.

A study in our laboratory compared follicle activity between mares of similar age with low and high body conditions.[48] Examinations began during the anovulatory season (August 14, Southern Hemisphere) and continued until the second ovulation of the year. Low body condition, compared to high body condition, was associated significantly with the following: longer interval to first ovulation; smaller diameter of the preovulatory follicle at maximum (45 vs. 51 mm, combined for both ovulations) and at Day -1 (45 vs. 50 mm); small diameter of the four largest follicles; fewer medium (11-19 mm) and large (≥20 mm) follicles; and fewer total number of follicles ≥5 mm. The body condition score was positively correlated with the preovulatory follicle at maximum diameter and at Day -1.

A recent study of spontaneous ovulatory waves in mares compared the effects of age (5 to 6 yr, young; 10 to 14 yr, intermediate; ≥18 yr, old) on follicle dynamics during an IOI (n = 46).[49] The old mares were not approaching senescence, as indicated by regular lengths of IOIs. The length of the IOI was approximately one day longer in the old group than in the younger groups and was attributable to slower growth rate of the ovulatory follicle and lower luteinizing hormone (LH) concentrations. The old

group had diminished follicle activity, as indicated by significantly smaller and fewer follicles. The diameters of the preovulatory follicle on Day -1 and at maximum were smallest in the old group. In an early study of age in mares, the preovulatory follicle grew more rapidly in young mares (5 to 7 yr) than in mares ≥15 yr.[50] The results of the recent study are also consistent with the finding of fewer follicles ≤20 mm in older ponies,[50,51] although some of the mares in the early reports were approaching senescence. These studies indicate the importance of age as a potential confounding factor for development of theriogenology programs (e.g., optimal time to breed and superovulation regimes) and in equine research protocols.

Color-Doppler ultrasonographic characteristics of the preovulatory follicle

Follicle wall blood flow and perfusion changes

Recently, transrectal Doppler ultrasonography has been utilized increasingly for research and clinical studies of ovarian and follicle hemodynamics in large farm animals.[52-55] Follicle blood-flow assessment by Doppler ultrasonography has been used in mares to study follicle selection,[5] anovulation during transitional seasons,[56] first versus later ovulations of the year,[43] follicle maturity and proximity to ovulation,[13,15,21,57] oocyte recovery rate, maturity, and quality,[58] effects of circulatory hCG antibodies on follicle and oocyte,[59] age-related effects,[14] and pregnancy establishment.[60,61]

The use of color-Doppler technology to evaluate the vascularity of the follicle wall in mares started in 2004. In a study of the vascular changes associated with the beginning of follicle deviation, the future dominant follicle was evaluated by transrectal color-Doppler ultrasonography until the follicle was about 30 mm, four days before ovulation.[5] The results demonstrated that deviation in the blood flow between the two largest follicles occurred one to two days before diameter deviation during follicle selection in mares. This conclusion is compatible with an earlier demonstration that an anechoic band surrounding the granulosa of the dominant follicle begins to expand differentially between the two largest follicles one day before the beginning of diameter deviation.[4] These results provided the first evidence in any species that differential blood-flow changes between future dominant and subordinate follicles begin early in the ovulatory wave and precede diameter deviation during follicle selection.

Color-Doppler ultrasonography also has the potential for judging the status (future ovulatory or anovulatory) of dominant follicles during the transitional period.[56,57] During the anovulatory transitional season, vascular changes in the follicle walls of both a future dominant anovulatory follicle and a future ovulatory follicle were studied from 25 mm until seven days after the follicle was 30 mm.[56] Blood-flow area was already lower for dominant-sized anovulatory follicles than for ovulatory follicles at the time blood-flow determinations began at 25 mm. A hypothesis for anovulation that involves hormones and follicle angiogenesis during the transitional period has been discussed elsewhere.[62] In this regard, preovulatory vascular changes have been compared between the first and later ovulations of the year in 40 pony mares for six days preceding ovulation.[43] Although follicle blood-flow area increased towards the ovulation day in both groups, results demonstrated that follicle vascularization and the LH surge were attenuated preceding the first ovulation of the year, with no indication that estradiol was involved in the differences between the first and later ovulations.

In regard to preovulatory follicle blood flow, recent studies have shown a daily increase in vascularity of the wall of the dominant follicle as it matures and approaches the day of ovulation.[13,14,43,57] However, on the day of ovulation, a few hours before evacuation an abrupt decrease in blood perfusion in the wall of the preovulatory follicle has been detected.[13,21]

Prediction of ovulation: preliminary results

The ability to control or predict ovulation time has practical implications for the equine industry and for research purposes and is a desirable goal. Currently, there are hormonal agents such as hCG, gonadotropin-releasing hormone (GnRH), and recombinant equine LH (reLH) that can induce ovulation in most (60 to 90%) mares within a predictable time after treatment (e.g., 24 to 48 hours); however, there is a proportion (10 to 40%) of mares that do not respond in a timely fashion to the administration of these hormones, and presumably ovulate on their own. Therefore, regardless of the use or disuse of hormonal

treatment, there are several practical situations that need the prediction of impending ovulation within 24 hours or even within a few hours. These scenarios can be exemplified by cases of horse breeder associations that allow only natural mating, clinics and farms that use cooled or frozen semen, limited semen quality or quantity as a result of stallion age or morbidity, assisted reproductive techniques, and research protocols. Therefore, the use of the main ultrasonographic signs of impending evacuation of the preovulatory follicle (see previous section) can be helpful to predict the time of ovulation in mares.

Few ultrasonographic studies of the preovulatory follicle have been done with a specific design to validate a methodology to predict the time of ovulation in mares. In a detailed study, echogenicity of the granulosa layer and prominence of an anechoic band beneath the granulosa reached a maximum score in 70% of mares on the day before ovulation.[6] The efficiency of these two echotexture characteristics was compared with follicle diameter as criteria for initiating breeding early in the ovulatory season. Results indicated that the ultrasonographic echotextural characteristics were superior to diameter in identifying the optimal breeding day in mares. Another study has concluded that the slopes of regression lines for the same previous characteristics were useful in predicting impending ovulation within 24 to 48 hours.[9] Recently, two studies were conducted to allow prediction of ovulation within different hours in hCG and non-hCG treated mares.[63] Prediction of ovulation in each experiment was carried out by two different operators without the knowledge of either the previous B-mode ultrasound scan record or day of the estrous cycle of each animal. In the first experiment, ultrasonographic scanning was done every six hours after a ≥35 mm follicle in four groups of mares. In the second experiment, the frequency of scanning was every 1, 12, or 24 hours after a ≥32 mm follicle. Attempted predictions based on each scan within each group for both studies were classified as correct or incorrect. The mean percentage of correct predictions for both experiments was 92%. However, in about 36% of the mares prediction could not be attempted due to insufficient follicular wall characteristics. These results, although limited by the number of animals, demonstrate that the degree of certainty for correct diagnosis can be high for independent operators; however, there are several mares that do not show the combination of adequate ultrasonographic follicular wall signs to be judged as impending ovulation. Therefore, studies to predict impending ovulation with B-mode and color-Doppler ultrasonography using different combinations of follicular wall characteristics (e.g., serration of granulosa and decrease in blood flow) are warranted.

Types of preovulatory follicle outcomes

Ovulation

Normal evacuation. As ovulation approaches, a bulge at the apex of the follicle can be detected at the ovulation fossa by laparoscopy[64] or by ultrasonographic imaging.[13,15,16] The apex is a thin-walled and relatively avascular portion of the preovulatory follicle[21] that separates an infundibular fluid pocket[65] from the follicular antrum. During follicle evacuation at ovulation the follicular fluid enters the infundibular fluid accumulation and the majority of the discharged follicular fluid is drained into the abdomen.

The first chance observations of continuous follicle evacuation by transrectal ultrasound were made in mares.[66] In subsequent planned studies, two distinctive evacuation patterns (abrupt and gradual) were observed during continuous monitoring with B-mode ultrasound.[22,67] In about 50% of ovulations, evacuation of follicular fluid from the preovulatory follicle was an abrupt process ranging from five to 90 seconds, with approximately 15% of the initial fluid remaining in the antrum. In the other 50%, release of follicular fluid was a slow and gradual process taking six to seven minutes to evacuate about 90% of the initial volume. Complete loss of detectable follicular fluid from the antrum and the extraovarian space usually takes minutes or hours, but may last as long as two days.[21,22] In some occasions, residual antral fluid may not be lost before blood or transudate begins to collect within the antral cavity. Evacuation can be suspected to be under way when the follicle is reduced in size and irregular in shape. Examination a few minutes later and sometimes a few seconds later, if early in the process, may indicate further evacuation and confirm that ovulation is under way or has occurred. Other reports[21,68] described evacuations that were considered atypical (see septated evacuation).

Septated evacuation. Follicle evacuations with atypical sites on the day of ovulation (estimated decrease in antrum) were compartmentalized with irregular echoic septa and contained apparent follicular fluid.[68] Whether complete evacuation occurred was not determined, owing to a daily interval between examinations. Atypical sites occurred in approximately 7% of hCG-treated and nontreated mares and were less common in young (3 to 7 years; 2%, 1/64) than in intermediate (15 to 19 years; 8%, 4/48), and old mares (≥20 years; 16%, 6/38). Preliminary observations in our previous research projects, based on hourly ultrasonographic examinations before ovulation, suggested that septated evacuations were prolonged and may be related to the location of blood vessels at the periphery of the follicle.

A recent study was performed using color-Doppler and B-mode film clips taken of the preovulatory follicle one hour or every hour during 12 hours preceding the beginning of evacuation in normal and septated evacuators.[21] Locations of serrated granulosa and color-flow signals were determined by clock-face positions with the apex of the follicle (future ovulation site) at 12 o'clock. Mares were divided into a group with normal follicle evacuation (completion within 1 hour; n = 21 mares) and a group with septated evacuations (completion of evacuation in ≥3 hours and formation of echoic trabeculae in the antrum during evacuation; n = 5 mares). The percentage of follicle circumference with color-flow signals was greater one hour before the beginning of evacuation in the septated group (76%) than in the normal group (37%). For mares with hourly data available before evacuation (n = 8), there was a greater decrease in percentage of follicle circumference with color-flow signals beginning six hours before ovulation in the normal group than in the septated group. In the normal-evacuation group, serration and blood-flow signals were located at the basal hemisphere of the follicle directly opposite to the apex. The apical area was devoid of both serration and color-flow signals. In the septated evacuation group, color-flow signals and serration were detected at every clock-face position in each mare at the hour before ovulation. Results supported the hypothesis that prolonged septated follicle evacuation is associated with vascularization and serration of a greater circumference of the follicle than for normal evacuation, and vascularization includes the apical area. The results also indicated that detectable blood-flow signals were lost from the apical pole over the few hours before ovulation in the normal evacuations but not in the septated evacuations. The mechanism involved in the loss of blood-flow signals from the apical area in the normal evacuations is unknown, but may have been associated with the approach of a narrower apex toward the ovulation fossa. These findings suggest that when a reduction in vascularization at a broad apex does not occur and ovulation occurs in the presence of apical vessels, the evacuation is prolonged and trabeculae form in the antrum during evacuation. Studies are necessary to evaluate if the oocyte is released from the follicle during prolonged septated evacuation and determine the fertility of this type of ovulation.

Hemorrhagic anovulatory follicle

Extravasation of blood into follicles, ovulation sites, and corpora lutea is common in mares.[34,40] A hematoma that forms in the antrum instead of ovulation has been termed a hemorrhagic anovulatory follicle (HAF).[66,69] Similar structures also have been termed hemorrhagic follicles,[34] anovulatory hemorrhagic follicles,[70,71] persistent anovulatory follicles,[72] and autumn follicles.[73] The economic importance of HAFs as a breeding-management problem in mares has been noted[72,74,75] and reflects anovulation of a follicle after the mare has been bred. The extent of the problem is indicated by a 5% and 20% incidence during the early and late ovulatory season, respectively (reviewed in Ginther et al. 2007[76]). Moreover, the syndrome seems to be more common in old mares and may occur repeatedly in an individual, sometimes encompassing much or all of the breeding season.

The morphology and vascularity of HAFs in mares and the endocrinology immediately preceding HAF formation have been studied in control and HAF groups.[77] The day of ovulation and the first day of HAF formation, as indicated by cloudiness of follicular fluid, have been defined as Day 0. The frequency of discrete gray-scale ultrasonic indicators of impending ovulation[15] and follicle diameter on Day -1 did not differ between future ovulating and HAF groups. On Day -1, the circumference of the follicle wall of future HAFs had more color-Doppler signals of blood flow than in the control mares. In regard to hormones, higher estradiol concentrations occurred a few days before HAF formation, but systemic LH,

follicle stimulating hormone (FSH), and progesterone were not altered during conversion of a preovulatory follicle into an HAF. In a recent comparative study of induced waves using prostaglandin F2α (PGF) treatment on Day 10 and ablation of follicles ≥6 mm and spontaneous ovulatory waves, an unexpected high incidence of HAFs (24%; n = 21) occurred in the induced waves and none in the spontaneous waves.[78] The induced ovulatory waves differed considerably from spontaneous waves, including greater LH concentrations during much of the induced wave and greater growth rate of a smaller ovulatory follicle. In this regard, results of another recent study[79] indicated that the high incidence of HAFs after PGF/ablation was associated with later follicle emergence and immediate and continuing greater LH concentration after PGF treatment, apparently augmented by an inherently high pretreatment LH concentration. A retrospective study[80] found a significant association between the use of prostaglandin analogues and HAF formation over a 20-yr period. Pharmacological induction of HAFs and LUFs in mares has been recently obtained by systemic treatment with flunixin-meglumine, a cyclooxygenase-2 (COX-2) inhibitor, given during the preovulatory period.[81,82] Furthermore, a recent histological and immunohistochemical study[83] was unable to determine conclusively the participation of several angiogenic factors or the LH receptor in HAF formation. Studies of HAF formation and the underlying mechanisms in mares may be of comparative importance for other animal species and women that have similar ultrasonographic structures.

Atresia

The process of atresia of a preovulatory follicle during an expeccted major ovulatory wave is not a common finding during the natural equine breeding season, but it occurs quite often during follicular waves in the spring and fall transitional seasons. A postulated hormonal mechanism for anovulation of a preovulatory follicle in mares has been proposed[62] and its effects may possibly lead to changes in the follicular wall that could be appreciated through ultrasonography during a transitional season.

Vascularity of the preovulatory follicle versus fertility

The degree of vascular perfusion of the ovary and follicles, assessed by color- or power-Doppler ultrasonography, has been used as a potential new technology for research and clinical studies of ovarian and follicle hemodynamics and to predict fertility in horses, cattle, and humans. Increased follicle blood flow, along with a rapid increase in LH at the terminal stage of follicle maturation has been associated with meiosis resumption and completion of oocyte maturation. Greater vascularity of the preovulatory follicle has been associated with greater follicle diameter (women,[84] mares,[60] heifers[61]), retrieval rate of oocytes (women,[84] mares,[58] heifers[86]), retrieval rate of mature oocytes (mares[58,59,85]), in vitro fertilization rate (women,[84] heifers[86]), pregnancy rate (women,[84,87-91] mares,[60] heifers[61]) and lower incidence of triploidy (women).[84] In addition, follicles with greater blood flow resulted in better embryos and more pregnancies after embryo transfer in women.[87,88,90,92,93]

The relationships of oocyte maturity, 30 hours after hCG treatment, to blood flow (color-Doppler mode) and ultrasonographic characteristics of impending ovulation (B-mode) of the preovulatory follicle have been recently studied in mares.[58] The vascularity of the preovulatory follicle tended to be greater for follicles that contained a mature oocyte. Follicles with an oocyte recovered had a significantly greater frequency of serration of granulosa and greater frequency of an apical area (indicators of impending ovulation). The frequency of serration of granulosa and decreased turgidity was greater in follicles that contained a mature oocyte. Results indicated that recovery of an oocyte and maturity of the recovered oocytes are both dependent upon the rate of follicle maturity in response to the hCG treatment.

Recent studies have tested the hypothesis that a higher pregnancy rate is associated with greater blood flow to the preovulatory follicle before breeding. The studies used color- and power-Doppler ultrasonography and indicated that pregnancy rates were greater in mares[60] and heifers[61] that displayed increased follicle blood flow. In both of these studies, mares and heifers were previously treated with hCG and GnRH, respectively, to induce ovulation. Follicle blood flow was evaluated at the time of treatment and natural breeding or artificial insemination. Although the results of both studies are preliminary, since they involved a limited number of animals, important statistical differences were found

between animals that became pregnant versus nonpregnant in each study. In the mare study,[60] B-mode echogenicity and thickness of the granulosa layer and prominence of the anechoic band beneath the granulosa increased similarly in both pregnant and nonpregnant groups. An increase in follicle diameter and percentage of follicle circumference with color-Doppler signals was greater between the time of hCG treatment (hour 0) and artificial insemination (hour 30) in the pregnant group than in the nonpregnant group. Spectral-Doppler measurements were made at the most prominent intraovarian color signal. Decreases in resistance and pulsatility indices were greater between hours 0 and 30 in the pregnant group than in the nonpregnant group, indicating increased vascular perfusion downstream from the spectral measurement in the pregnant group. Relative peak systolic velocity and time-averaged maximum velocity of blood flow at the point of spectral assessment was greater in the pregnant group.

Aiming to evaluate fertility with a different perspective, a recent study in mares[59] investigated the effect of an ovulation-inducing dose of hCG in the presence versus absence of hCG antibodies on blood flow of the preovulatory follicle and maturity and quality of recovered oocytes at 30 hours post-treatment. The percentage of the follicle wall with blood-flow signals was less in the antibody positive group than in the negative group. The oocyte recovery rate (62%, 37/60) between hCG antibody-positive (44%) and negative mares (68%) tended to be different. The antibody-positive group had fewer mature (MII) and more atypical oocytes than the antibody-negative group.

Although preliminary, our recent studies in mares supported the hypothesis that greater blood flow to the preovulatory follicle is associated with higher follicle and oocyte maturation rates, oocyte recovery and quality rates, and pregnancy rates. Similar results were found in the heifer study,[61] demonstrating that highly vascularized preovulatory follicles are more likely to be associated with higher pregnancy rates, as previously seen for mares and women.

Relationships between preovulatory follicle and corpus luteum blood flow in mares

In a recent study,[94] the diameter and blood flow of the preovulatory follicle were greater ($P<0.05$) than for those follicles that underwent atresia in single and double ovulator mares. The preovulatory follicle diameter and the blood flow were positively correlated in ovulatory ($r=0.51$; $P<0.0001$) and in atretic ($r=0.32$; $P<0.02$) follicles. In double ovulator mares, preovulatory follicle diameter and blood flow increased ($P<0.0006$) during five days before ovulation with no difference between the two follicles in the same cycle for each parameter. The preovulatory follicle blood flow was positively correlated ($r=0.32$; $P<0.0009$) with CL vascularity during the first periovulatory period (Days -7 to +6) of the season. Furthermore, a positive correlation ($r=0.58$; $P<0.01$) was obtained between the maximum vascularity of the preovulatory follicle and its subsequent CL. The results of this study demonstrated that: 1) potential atretic preovulatory follicles had low blood flow; 2) double preovulatory follicles had similar vascularity; and 3) greater preovulatory follicle blood flow was associated with higher CL vascularity.

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Endocrinopathic laminitis and equine metabolic syndrome

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Abstract

A common feature of equine laminitis is the dysfunction and failure of the dermal-epidermal attachment between the hoof wall and the distal phalanx, resulting in compromised digital support. Conditions that cause insulin resistance (IR) in horses, including obesity, equine metabolic syndrome (EMS), and pituitary pars intermedia dysfunction (PPID), greatly increase the risk of endocrinopathic laminitis (EL). Equine and human metabolic syndrome share some common features, including insulin resistance, regional adiposity, a pro-inflammatory state, and inflammatory and degenerative co-morbidities, with laminitis being the predominant co-morbidity in the horse. Epidemiological studies have identified many negative effects of IR and obesity on conception and pregnancy outcome in women. Studies are needed to determine the effects of IR on equine fertility. Broodmares might be at particular risk for EL due to the physiological decrease in insulin sensitivity associated with pregnancy. Previous laminitic episodes, insulin resistance due to obesity, EMS, or PPID, painful or stressful conditions, septic conditions, lack of exercise, exogenous corticosteroid administration, grazing high fructan content pasture, and high glycemic index feed supplementation should all be considered potential risk factors for EL that could have an additive effect with pregnancy-associated IR to push mares over the hypothetical insulin concentration threshold for laminitis induction. Strategies for the management of at-risk mares include diagnosis and treatment of EMS and PPID, weight and glycemic response management through diet and exercise, and therapeutic hoof trimming for mares with stable chronic laminitis. Mares with unstable chronic laminitis and/or inadequately controlled IR should be considered unfit for breeding.

Keywords: laminitis, insulin resistance, metabolic syndrome, equine, obesity

Equine lamellar anatomy and laminitis

As shown in Figure 1, the delicate structure of the epidermal and dermal lamellae of the normal equine foot greatly increases the surface area of dermal-epidermal attachment and forms the suspensory apparatus of the distal phalanx, facilitating the transfer of the horse's weight from the skeletal elements of the digit to the hoof wall.[1] Each foot has 550-600 parallel primary epidermal lamellae (PELs), each of which has 150-200 secondary epidermal lamellae (SELs) with an estimated total surface area for lamellar attachment of approximately 0.8 m^2 per foot.[1,2] The reader is referred to other sources for more detailed information on the anatomy of this region.[1,3,4]

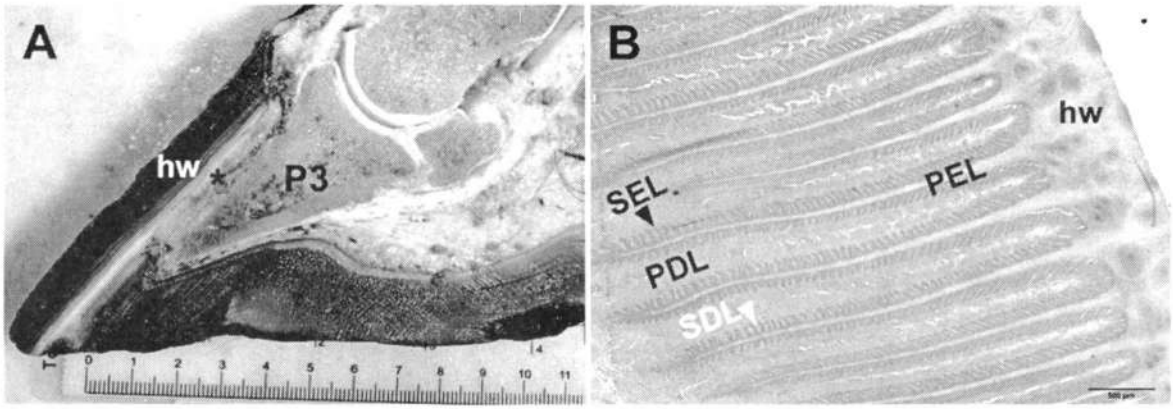

Figure 1: Normal equine hoof lamellar anatomy and microanatomy. A) Mid-sagittal section of a non-laminitic equine foot. Approximately 500 primary epidermal lamellae (*) run parallel to the inner aspect of the stratum medium, or hoof wall (hw) of each foot and interdigitate with the dermal lamellae and connective tissue of the hoof corium, transferring the weight of the horse from the distal phalanx (P3) to the hoof wall. Scale is in cm. B) Histological transverse section of the lamellar region stained with hematoxylin and eosin. The cut edge of the nonpigmented hoof wall (hw) is to the right and is continuous with the cornified primary epidermal lamellae (PEL). Each PEL has approximately 150 secondary epidermal lamellae (SEL) which greatly increase the surface area of the lamellar epidermal-dermal interface. The PELs and SELs interdigitate with primary dermal lamellae (PDL) and secondary dermal lamellae (SDL), which contain the vascular, nervous, and connective tissue. Scale bar = 500 μm, image courtesy of Julie Engiles, Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania.

Equine laminitis is a severe, debilitating and common disease of horses, often leading to the humane destruction of affected animals due to chronic pain and loss of use, and is recognized as a high priority topic for research by the United States Department of Agriculture and the American Association of Equine Practitioners.[5,6] Several conditions, which have been recently reviewed, are associated with the development of laminitis, including alimentary carbohydrate overload,[7] septic conditions,[8] black walnut extract toxicity,[9] excessive weight bearing or concussive forces to the foot,[10] and the focus of this review, endocrinopathies resulting in IR and hyperinsulinemia (HI).[11-14] Laminitis associated with IR or HI is collectively referred to as EL and is believed to include pasture-associated laminitis.[11] In all forms, the initiating insult sets off a clinically silent developmental phase that is followed by acute laminitis.[15] Signs of acute laminitis include shifting weight lameness, increased heat in affected feet, and bounding digital pulses. Once signs are present, lamellar damage may already be underway and the epidermal-dermal interface is weakened.[16] The hallmark of laminitis is the loss of dermal-epidermal attachment between the epidermal lamellae of the inner hoof wall and the interdigitating dermal lamellae of the underlying distal phalanx.[17] As shown in Figure 2, lamellar failure can result in displacement of P3 within the hoof capsule (failure of the digital suspensory apparatus) and chronic laminitis, or "founder".[1,15] Because the weight of the horse is primarily supported by the hoof wall via the lamellar attachment, unstable chronic laminitis causes secondary pathologies such as crushing of vasculature and neural tissue enclosed within the hoof capsule, osteolysis and fracture of the distal phalanx, and in the most severe cases, herniation of the distal phalanx through the sole of the hoof.[1,18,19]

The lamellar epidermal cells undergo a wound healing response during laminitis that results in the generation of "lamellar wedge" or ectopic white line cornified epidermal tissue.[19,20] This dysplastic tissue does not replace the mechanical functions of the original lamellae, might aggravate associated bone, vascular, and neural pathologies, and does not provide adequate barrier protection against ascending bacterial infections.[18,19] We have demonstrated that the dysplastic lamellae in horses with chronic laminitis and a lamellar wedge is deficient in a marker of epidermal stem cell proliferative capacity, consistent with the abnormal growth and differentiation of this tissue.[21] It is clearly preferable to prevent laminitis rather than to treat it.

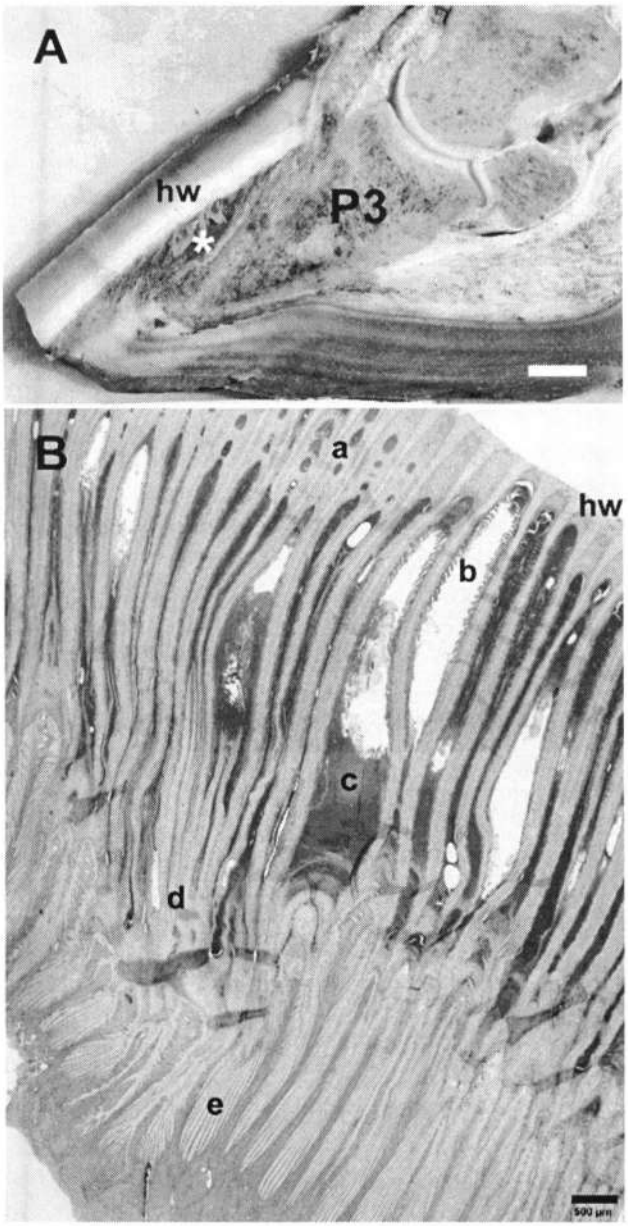

Figure 2: Pathological equine hoof lamellar anatomy and microanatomy from a 150 day pregnant mare with suspected equine metabolic syndrome and endocrinopathic laminitis of 8 week duration. A) Mid-sagittal section of laminitic front foot. The distal phalanx (P3) is displaced or rotated relative to the dorsal hoof wall (hw) and the lamellae are forming a dysplastic lamellar wedge in response to tissue damage (*). Weight-bearing is largely shifted from the hoof wall to the sole causing tissue damage and pain. Scale bar = 1 cm. B) Histological transverse section of the mid-dorsal lamellar region from the foot shown in (A) stained with periodic acid-Schiff/hematoxylin. The cut edge of the hoof wall (hw) is to the upper right. New cap horn tubules are forming between the PELs adjacent to the hoof wall in an attempt to fill the defect created (b) by dermal-epidermal and epidermal cell-cell detachment. Detached, necrotic SELs are apparent at (b) and serous exudate (c) fills the space, providing an excellent environment for bacterial culture and abscess formation. The hugely elongated PELs (d) and SELs (e) give rise to the dysplastic and hyperplastic cornified epidermal tissue of the lamellar wedge. Scale bar = 500 μm, image courtesy of Julie Engiles, Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania.

Insulin resistance, inflammatory co-morbidities, and endocrinopathic laminitis

Many cases of equine laminitis are associated with insulin resistance and an equine metabolic syndrome that has many parallels to human pre-diabetic metabolic syndrome.[14,22] Human metabolic syndrome, or syndrome X, is characterized by central obesity, atherogenic dyslipidemia (low high density lipoprotein-cholesterol (HDL-C), elevated low density lipoprotein-cholesterol (LDL-C) and serum triglycerides), a prothrombotic state, a pro-inflammatory state, hypertension, and IR, often culminating in β-cell failure, impaired glucose tolerance, and type 2 diabetes.[23,24] Decreased insulin sensitivity or increased IR is defined as the decreased biological response of a nutrient to a given concentration of insulin at the target tissue, e.g. liver, muscle, or adipose tissue.[25] Obesity is the most common risk factor related to IR. The list of human metabolic syndrome co-morbidities has grown in recent years and includes cardiovascular disease,[26] hepatic steatosis,[27,28] intramyocellular lipid accumulation,[28] sleep apnea,[29] osteoarthritis,[30] psoriasis,[31] and dementia and neurodegenerative diseases.[32] A common feature of these co-morbidities is inflammation.

There are significant data supporting an association between insulin resistance and HI and equine laminitis.[33-35] Insulin resistance in horses and ponies is typically associated with equine metabolic syndrome (EMS), equine Cushing's syndrome/PPID, or corticosteroid treatment.[14,36-38] Pituitary pars intermedia dysfunction is characterized by varying degrees of the following: excess adrenocorticotrophic hormone (ACTH) and cortisol, hirsutism, polyuria/polydipsia, weight loss, muscle wasting, an abnormal fat distribution, insulin resistance, and high (>50%) incidence of laminitis.[39] Equine metabolic syndrome is associated with regional adiposity with or without obesity.[40] Horses and ponies with EMS are typically "easy keepers" that gain and retain weight easily and include over-represented "thrifty" breeds (ponies, Morgans, Paso Finos, Arabians, Saddlebreds, Quarter Horses, Tennessee Walking Horses, and domesticated Spanish mustangs).[22,40] Regional adiposity commonly includes the neck ("cresty neck"), rump and tail head, and sheath in geldings or udder in mares.[22,40] A "cresty neck" score has been developed for ponies and correlated with HI, hyperleptinemia, and an increased risk of pasture-associated laminitis.[41,42] Obesity, increased cresty neck score, and HI have also been used to predict pasture-associated laminitis in horses.[35]

Although these studies and clinical impressions are compelling, there is a need for more rigorous epidemiological data on risk factors for laminitis. A current epidemiological study at the University of Minnesota (http://www.cvm.umn.edu/equinegenetics/ems) is attempting to provide a more thorough phenotypic characterization of EMS and laminitis risk. A separate study initiated by the American Association of Equine Practitioners (AAEP) Research Foundation is generating epidemiological data on risk factors for pasture and endocrinopathy-associated laminitis (http://www.vetmed.tamu.edu/vmth/laminitis). It is likely that various factors that influence plasma insulin concentration or IR have an additive effect on a horse's overall risk of EL.[11] These factors may include those that lower the hypothetical threshold for laminitis induction, such as lamellar damage due to previous laminitic episodes or abnormal hoof conformation, and those that increase insulin concentrations toward the threshold. The latter may include IR due to obesity, EMS or PPID, painful or stressful conditions that increase the insulin counter-regulatory hormones (cortisol, epinephrine, norepinephrine),[43] grazing high fructan content pasture and/or high starch feed supplementation,[11,44] septic conditions,[45] lack of exercise,[46] exogenous corticosteroid administration,[36] and pregnancy.[47] The ongoing epidemiological studies will provide data regarding some of these putative risk factors.

A common feature of human metabolic syndrome, EMS, and PPID is IR and a pro-inflammatory state. Humans tend to progress to type 2 diabetes, but horses nearly always compensate for IR with increased β-cell insulin production resulting in HI, although diabetes mellitus also occurs in horses resulting in persistent hyperglycemia with glucosuria.[22] Recently, studies utilizing a prolonged HI/euglycemic clamp, or HI model of experimental laminitis, have demonstrated that elevated insulin levels can induce laminitis in 48 h and 72 h in horses and ponies, respectively, by an unknown mechanism.[48,49] The "inflammation hypothesis" of human metabolic syndrome states that obesity results in a shift of the leukocyte population in adipose tissue to pro-inflammatory CD8+ T cells and M1 or activated macrophages resulting in pro-inflammatory adipocytokine production.[24,50] These factors impair insulin signaling, resulting in insulin resistance in adipose tissue, skeletal muscle and liver.[23] Adipocytokines also create a chronic inflammatory environment that contributes to the generation of inflammatory and degenerative co-morbidities in humans. Two studies have reported increases in serum pro-inflammatory cytokines in horses with increased body condition score and percent body fat.[51,52] In addition, impaired glucose transporter 4 (GLUT4) trafficking has been detected in muscle and adipose tissue from IR horses, consistent with impaired insulin signaling in these horses.[53,54] Moreover, acute HI of only 6 h duration increased circulating plasma tumor necrosis factor (TNF) protein concentration and increased peripheral blood leukocyte IL-6 and IL-1β mRNA expression, consistent with the activation of pro-inflammatory cytokine production.[55] In horses, the feet might be the organ system that is the most sensitive to this inflammatory environment, although the horses and ponies used for the HI model were not obese, and a more direct, toxic effect of the supraphysiologic serum insulin concentrations utilized for the model is implicated. The pathophysiology of HI laminitis and its relevance to naturally-occurring endocrinopathic laminitis are poorly understood, but could result from the activation of inflammation

and/or cell proliferation. Proposed theories for HI-induced laminitis etiopathogenesis include vascular effects,[12,48,56] activation of inflammation,[55] disturbance in glucose metabolism,[57,58] and direct insulin toxicity.[48,59] A possible mechanism for insulin toxicity is via activation of insulin-like growth factor-1 receptor (IGF-1R), resulting in the promotion of epidermal hyperproliferation and cell survival.[60-62]

Insulin resistance and reproduction: Effect of pregnancy on insulin sensitivity

Broodmares might be at particular risk for endocrinopathic laminitis due to the physiological effects of pregnancy on glucose and insulin dynamics. Mares with pre-existing IR or lamellar damage due to prior episodes of laminitis might be at or near the hypothetical HI threshold for induction of laminitis.[11] Pregnancy in women and dogs is associated with a 60% and 43%, respectively, decrease in insulin sensitivity and an increased insulin response to glucose.[25,63] This physiological IR of pregnancy facilitates glucose delivery to the fetus and is caused by the insulin counter-regulatory and post-receptor actions of progesterone, estradiol, growth hormone, placental lactogen, and placental cytokines.[63] In women, elevated cytokine and lipid concentrations during pregnancy are believed to induce the IR of pregnancy through a post-receptor mechanism: defects in the intracellular insulin signaling pathway resulting in decreased ability of insulin to mobilize GLUT4 from the cytoplasm to the plasma membrane.[25] Obese and overweight women are at increased risk for metabolic dysregulation in pregnancy resulting in gestational diabetes, preeclampsia, and fetal overgrowth.[25] Pregnancy is considered a metabolic stress test for the future risk of metabolic syndrome in women.[25] It could be very informative to determine if, likewise, the insulin response to oral glucose tolerance test during equine pregnancy has any correlation with later development of EMS and/or laminitis in broodmares.

A recent study by George et al detected decreased insulin sensitivity and glucose effectiveness and higher acute insulin response to glucose in pregnant (28 weeks gestation) vs. nonpregnant Thoroughbred mares.[47] The pregnant mares had prolonged hyperglycemia and HI in response to meal feeding that was particularly apparent following a high starch feed.[47] Insulin resistance was not significant later (48 weeks gestation), although this may have been due to non-insulin-mediated glucose disposal from the mother to the growing fetus through placental facilitated diffusion of glucose, which results in an overestimation of maternal insulin sensitivity, particularly in late gestation.[25] For mares that have underlying IR prior to pregnancy, the additional metabolic stress of pregnancy could greatly increase the risk of laminitis, although epidemiological studies are needed to verify this suspected risk. The laminitis case example shown in Figure 2 is from an obese mare that became acutely laminitic at approximately 90 days gestation and was eventually euthanized eight weeks later due to unstable chronic laminitis and unmanageable pain. The additive effects of obesity and pregnancy on IR might have initiated laminitis in this mare, and could possibly have been averted by weight management prior to breeding and by avoiding high starch feed and allowing exercise during pregnancy.

Insulin resistance and reproduction: negative effects on fertility

Not long ago, it was common practice to treat any overweight mare with perceived subfertility for hypothyroidism. The prevalence of hypothyroidism in adult horses is now believed to be very low.[64] Many of the clinical signs previously attributed to equine hypothyroidism, which included obesity, regional adiposity, and weight gain on little feed, are now associated with EMS.[40,64] In fact, horses that were made hypothyroid by surgical thyroidectomy or pharmacological suppression did not develop obesity or laminitis.[64] In addition, two studies were unable to document any association between hypothyroidism and infertility in mares and thyroidectomized mares are capable of conceiving and carrying foals to term.[65-68] Although previous over-diagnosis of hypothyroidism as the reason for treating mares with thyroid hormone supplementation may have been faulty, there is evidence that this therapy is useful, in combination with dietary restriction and exercise, in accelerating weight loss and improving insulin sensitivity.[69-71]

In defining EMS, Johnson stated, "Affected broodmares sometimes exhibit abnormal estrous cycling, and are notoriously difficult to breed successfully".[40] However, few published studies have documented a link between EMS or obesity and reproductive abnormalities in mares. Mares with high

body condition score (BCS)[72] are more likely to have continued estrous cyclicity and follicular activity during the winter compared to lean mares.[73-76] Gentry et al reported increased winter serum luteinizing hormone (LH) concentration and response to gonadotropin releasing hormone (GnRH) in the high BCS mares, but no difference in weekly LH, follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), growth hormone (GH), glucose or insulin during the autumnal transition and winter anovulatory period.[74] Vick et al reported significantly longer intervals between ovulations and prolonged luteal phases in obese mares (BCS = 7.5-9.0 on a scale of 1-9) compared with feed-restricted, previously obese mares (BCS = 4.0-5.0) during the late summer and autumn transitional period.[76] In contrast, Waller et al did not detect any effect on ovarian activity, day of ovulation, and gonadotropin and progesterone concentrations for high BCS mares with hyperleptinemia, although the control mares also had high BCS in that study.[75] A study using an experimental IR induction model resulted in no change in serum LH concentration or duration of luteal phase in treated mares, but did increase the interovulatory period and peak serum progesterone concentration.[77] Information on the effect of high BCS or IR on hormone concentrations and reproductive characteristics during the breeding season, especially for older mares with naturally-occurring EMS, is lacking.

The effect of obesity on fertility in women has been much more thoroughly documented and readers are referred to a recent review.[78] Obese women are three times more likely to suffer infertility then women with a normal body mass index.[79] Proposed negative impacts of obesity on infertility include control of ovulation, oocyte development, embryo development, endometrial development, implantation, and pregnancy loss.[78] Obesity, particularly central obesity, is associated with perturbations to the hypothalamic-pituitary-ovarian axis, menstrual cycle disturbance, oligo-/anovulation, and impaired response to assisted reproduction technologies.[78] Insulin acts on the ovary via the IGF1 receptor to stimulate ovarian steroidogenesis and LH receptor upregulation.[80] Insulin also enhances the sensitivity of pituitary gonadotroph cells to the action of Gn RH and modulates the bioavailability of the sex steroids via inhibition of hepatic sex hormone-binding globulin (SHBG) synthesis. Insulin resistance and HI leads to reduced SHBG, hyperandrogenemia, and disturbed IGF functionality, leading to menstrual and ovulatory disturbances.[78] Studies are needed to determine the effects of EMS, IR and HI on mare fertility. When considered along with the risk of laminitis, potential effects on fertility provide additional impetus to aggressively treat and control EMS in mares prior to breeding and during pregnancy.

Management of mares that are at risk for endocrinopathic laminitis

Equine metabolic syndrome and EL risk should be managed through diagnosis of pre-existing laminitis, BCS and regional adiposity evaluation and monitoring, blood testing for endocrinopathic changes, and aggressive weight and serum insulin concentration normalization through diet, exercise, and, for refractory cases, thyroid hormone supplementation. Ideally, weight and insulin normalization should occur prior to breeding since the addition of pregnancy-associated IR to underlying IR could push the mare over the hypothetical insulin concentration threshold for EL.

As part of the breeding soundness evaluation or annual wellness examination should include screening for EMS and EL. Pre-existing, undetected laminitis is quite common, particularly in horses that are not being used for athletic endeavors, such as broodmares.[46] Physical examination of the feet of these horses might detect the following: divergent laminitic growth rings (founder lines), dropped of flat sole(s), solar abscesses, widened white line at the toe, seedy toe, and/or sensitivity to hoof testers at the toe. Horses with suspected EMS and/or EL should have a carefully aligned lateromedial radiograph of the foot taken to determine if displacement of P3 within the hoof capsule has occurred, to serve as a baseline radiograph, and to guide decisions regarding corrective trimming and shoeing. BCS should be evaluated using the AAEP BCS score developed by Henneke et al, that ranges from 1 (poor) to 9 (obese).[72] Horses with BCS $\geq$ 7/9 are at risk for EMS and EL. It is also possible to estimate body weight in pounds by measuring the heart girth and body length (point of shoulder to point of buttock) in inches and applying the formula (heartgirth x heartgirth x body length)/330 = weight in pounds.[46] Regional adiposity can be detected as cresty neck, enlarged udder, and fat around the tail head. Cresty neck can be assessed and monitored using the cresty neck score with a score of $\geq$ 3/5 suggestive of EMS,[41,42] or by

dividing the distance along a line from the poll to the cranial aspect of the withers (x) by 4 and measuring the neck circumference at 0.25x, 0.5x, and 0.9=75x.[22]

Blood testing should be performed on any horse with a history and physical findings consistent with EMS or PPID. A fasting screening test can be performed by leaving one flake of hay after 10:00 p.m. and collecting blood in the morning prior to feeding. Pituitary pars intermedia dysfunction is suspected if ACTH is >35 pg/ml, although elevated values are normal during the period of decreasing day length (August, September, October in the northern hemisphere),[81] and horses with elevated levels at that time can be treated with pergolide and then re-evaluated in November after a two week withdrawel from pergolide.[46] A diagnosis of EMS is supported by a fasting insulin concentration >20 μU/ml and fasting leptin concentration > 7 ng/ml, although the latter assay is not commercially available at this time.[22] An elevated fasting glucose >110 mg/dl is also consistent with EMS or could be evidence of diabetes mellitus. Clinical signs and these fasting values are adequate for diagnosis of EMS, but if the results are equivocal, dynamic testing using a combined intravenous glucose-insulin test or oral sugar test, as described,[22] should be performed.

Equine metabolic syndrome, PPID, obesity, and HI should be controlled prior to breeding due to the risk of EL. Pituitary pars intermedia dysfunction is treated with pergolide starting at 1 mg/day orally and rechecking serum ACTH and insulin after one month, the goal being ACTH < 70 pg/ml with a dosage range of 1-5 mg pergolide/day. Obesity and EMS should be managed by limiting grazing with a grazing muzzle or limited turn-out and avoiding pasture in the spring and fall when the non-structural carbohydrate (NSC) content is the greatest.[44] A weight loss regimen should be instituted that includes feeding a grass hay with analyzed NSC content of less than 10% (dry matter basis) fed at 1.5% of ideal body weight per day with vitamin and mineral supplementation and reduced to 1% of ideal body weight if no weight is lost after one month.[22] Exercise promotes weight loss and improves insulin sensitivity and should be part of the program.[82] If the horse cannot be ridden or lunged, at the very least, it should have turn out in a small grass paddock with a companion since social interaction increases activity.[22] Ideally, exercise consisting of a minimum of 30 min at a trot or canter, four to seven days/week, should be instituted.[22,46,82] For lame horses, some benefit can be derived from hand walking.[46] Obviously, the pregnant broodmare presents a difficult challenge since feed supplementation is indicated at least for the last trimester to support rapid fetal growth at that time.[83] Based on the findings of George et al, a high fat/high fiber feed is recommended for broodmares with EMS to reduce the glycemic and insulinemic response, although more research is needed on this topic.[47]

If the horse is either refractory to the weight loss regimen or is perceived to be at high risk of, or already suffering from EL, thyroid hormone supplementation with levothyroxine can be instituted as an aide for weight loss and to normalize insulin concentrations more rapidly.[22,46] Short term treatment (three to six months) is recommended at 0.1 mg/kg or 48 mg/day for horses weighing 450-525 kg.[22]

Due to the potential catastrophic consequences of EL during pregnancy, the veterinarian and owner must work together to manage obesity and EMS and prevent EL. Owners should be strongly urged not to breed mares with existing obesity, endocrinopathy, or EL until weight and insulin concentrations have been normalized and the laminitis is stabilized.

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Integration of advanced assisted reproduction in the equine practice

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Abstract

Assisted reproductive technologies (ART) have been developed for the horse and can aid in the production of offspring from mares or stallions that are subfertile or infertile using standard breeding procedures or embryo transfer. Many ART procedures require special equipment or expertise, limiting their distribution in veterinary practices. However, as equine ART continue to develop, the equine practitioner has opportunities for new clinical endeavors and referral relationships. Established procedures for equine ART will be reviewed, with emphasis on clinical relevance, and the potential for ART in equine practices will be discussed.

Keywords: Equine, ART, oocyte, oocyte transfer, ICSI

Oocyte collection

The basis of many ART procedures is the collection and manipulation of equine oocytes.[1] Oocytes can be collected from the maturing or immature follicles of live mares or from the harvested ovaries of deceased mares.

Oocytes collected from maturing follicles are typically collected from the dominant follicle of the estrous mare between two and 18 h before the expected time of ovulation. Maturation of the follicle and oocyte is often induced by the administration of a gonadotropin releasing hormone (GnRH) analog and/or human chorionic gonadotrioin (hCG) after the follicle reaches > 33 mm in diameter. Most oocytes are at Metaphase I, when collected at approximately 12 h prior to the anticipated ovulation time, and at Metaphase II when collected within a few hours of the anticipated time of ovulation. Oocytes collected 12 h before the anticipated time of ovulation are cultured in vitro for 12 to 16 h before transfer or sperm injection to allow the completion of maturation.[2-4] Because maturation is induced in vivo, the addition of hormones to the culture medium is not required.

Oocytes are easier to collect as the follicle approaches maturation, and the attachments of the granulosa and cumulus cells loosen. When oocyte collections were performed in our laboratory by personnel experienced in palpation techniques, but with no previous experience in transvaginal oocyte recoveries, oocyte collection rates were significantly higher when collection attempts were done at approximately 36 versus 24 h after hCG and deslorelin (65% versus 38%, respectively; unpublished data). However, with experience at the technique, oocyte collection rates between 70 to 80% are expected.[5]

Procedures to collect oocytes are not overly difficult, but they do require practice for proficiency and training of support personnel. Minimal equipment is needed if oocytes are collected from large, preovulatory follicles through the mare's flank.[6] A trocar is used to place a cannula through the flank. The cannula will be used as a needle guide, as the ovary is manipulated per rectum. An advantage to the flank approach is that the needle does not puncture the vaginal wall of mares with chronic or severe uterine infections and potential vaginal contamination. The most common procedure for oocyte collections is transvaginal, ultrasound-guided follicular aspirations.[1] This method requires an ultrasound console and probe; linear, curvilinear and sector probes have been used. The probe is placed in a casing containing a needle guide and positioned within the anterior vagina. The ovary is manipulated per rectum to position the follicle, while the procedure is imaged on the ultrasound monitor.

In most mares, the preovulatory follicle is relatively easy to position, and the needle can be used to puncture the follicular antrum while avoiding adjacent structures, such as the oviduct or broad ligament. When oocytes are collected from the dominant follicles of estrous mares, only one or two oocytes are available per cycle. The mare can be short cycled through the use of prostaglandin at five or six d after the oocyte collection, resulting in oocyte collections at approximately two-week intervals. Oocyte collections have to be scheduled to assure the follicle will not ovulate before collection of the oocyte. Scheduling of oocyte collections is dictated by follicular development and availability of semen.

Often, a window of only a few days is available to reliably induce follicular maturation and collect the oocyte prior to initiation of oocyte maturation in vivo and subsequent ovulation.

Oocytes can also be collected without exogenous initiation of maturation and from the entire population of follicles on the ovary. This provides more follicles and, potentially, more oocytes per collection. The immature oocyte is closely adhered to the follicular wall within a hillock of cumulus cells. Oocyte collections are done using the ultrasound-guided technique to image and position the small follicles for needle punctures.[6-8] Because the oocyte is closely adhered to the follicular wall, the follicle is usually repeatedly flushed and scraped. Oocyte collection rates (per follicle) vary with techniques and individual mares, but oocyte collection rate from immature follicles is often approximately half of the expected rate from preovulatory follicles. Oocytes can be collected at 10- to 15-d intervals.[6-8] Hormones and growth factors are usually added to the maturation medium, with oocytes matured between 24 and 30 h.[6-8] The viability of oocyte can vary with the different follicles and stages of development. Although we anticipate an almost 100% maturation rate (extrusion of a polar body with maturation to Metaphase II) for oocytes collected from preovulatory follicles, the maturation rate of oocytes collected from immature follicles is approximately half. Collection of oocytes from small follicles requires numerous punctures over the ovarian surface. Consequently, it is more difficult to avoid structures adherent to the ovary, such as the oviduct or broad ligament.

Identification and handling of oocytes

The identification and handling of oocytes is important for the success of subsequent procedures. In general, oocytes are more sensitive to temperature changes than embryos. The temperature of media and equipment should be carefully maintained at approximately body temperature, and fluctuations in temperature should be avoided. Collections from maturing follicles are not filtered in our laboratory. The flush medium contains granulosa cells that have become mucoid during maturation and will adhere to the filter and prevent media flow. The follicular flush from preovulatory follicles usually contains blood, as the complex vasculature network surrounding the follicle is disrupted. Identification of the maturing oocyte is facilitated by the clear mass of cumulus cells surrounding the oocyte.[1] The oocyte is often polarized in appearance with a surrounding ring of cells, the corona radiata, in a very clear mass of cumulus cells that are translucent in appearance. Large numbers of granulosa cells can be obtained; they are usually in sheets, with a slightly dark and grainy appearance when compared to cumulus cells.

Follicular fluid and flush medium collected from small follicles can be filtered to help in finding the associated oocytes. Embryo collection filters can be used, but the mesh has to be carefully rinsed to remove the follicular cells and oocytes. Immature oocytes are often surrounded by a compact mass of cumulus cells which are difficult to differentiate from granulosa cells; therefore, care must be taken to properly identify the oocyte. After identification, oocytes should be quickly rinsed in appropriate medium and placed into culture.

Oocyte collections in a clinical practice

It is feasible to do oocyte collections in a clinical setting. However, conditions should be carefully controlled to assure cleanliness and attention to detail. Although infrequent, complications reported after oocyte collections include fatal hemorrhage,[9] peritonitis[10] and a thickening of the ovarian serosa.[6] Careful sedation and handling of the mare and ovarian manipulations are important to avoid problems during oocyte collections.[10] Equipment and procedures to handle oocytes need to be prepared in advance, as even short intervals of altered temperature or less than ideal media conditions can be deleterious to the oocyte.

Oocyte transfer

Oocyte transfer involves the collection and transfer of an oocyte from a donor mare into the oviduct of an inseminated recipient. Fertilization, embryo and fetal development occur within the recipient. Oocyte transfer is primarily used to obtain pregnancies from mares that have severely reduced fertility because of various problems within the reproductive tract, such as cervical, uterine or oviductal

pathology.[5,11,12] However, the donor mare must still cycle and produce viable oocytes. The oocyte is transferred into the infundibular os of the oviduct during a standing flank laparotomy to expose the ovary and oviduct. In our laboratory, the recipient is inseminated 12 to 18 h prior to transfer and again at two or three h after transfer if a second dose of semen is available. When mares were inseminated before and after oocyte transfer with semen from two fertile stallions, 92% of the oocytes were fertilized from the insemination prior to transfer.[3] We prefer to inseminate at least one billion progressively motile sperm to optimize the success of oocyte transfer. The transfer of preovulatory oocytes from the donor to recipient is the least manipulative ART procedure to produce pregnancies from otherwise infertile mares, as oocyte maturation is initiated in vivo, and fertilization and embryo development occurs within a mare's reproductive tract. However the procedure is not effective for subfertile stallions.

For oocyte transfer, ovulation of a donor mare's follicle has to be avoided to prevent fertilization of the incorrect oocyte. Different types of recipients have been used. Noncyclic or early estrous mares or mares with hormonally suppressed ovarian activity can be induced into estrus with the administration of exogenous estrogen while only small follicles are present on the ovaries; after transfer, progesterone or a synthetic progestin is administered to maintain the pregnancy. Cyclic mares can also be used after the collection of their own oocyte from their preovulatory follicle. In our laboratory, pregnancy rates after oocyte transfer are not different for cyclic or noncyclic mares.[5]

Oocyte transfer is feasible under controlled clinical conditions. Although laparoscopic methods have been attempted, most transfers are done by standing flank laparotomy. The surgeon exposes and holds the ovary, while a tehnician manipulates the transfer pipette into the oviduct and expels the oocyte. Pregnancy rates are not different for oocytes cultured for the completion of maturation or collected just prior to ovulation and directly transferred into recipients' oviducts.[4] Collection of oocytes from preovulatory follicles and immediate transfers negate the necessity of equipment for oocyte culture. However, the oocyte must be maintained in proper medium and temperature conditions to maintain developmental potential even if it is only held outside of the mare for a short interval of time.

Gamete intrafallopian transfer and intracytoplasmic sperm injection

Although oocyte transfer is quite successful when good quality semen is available, other procedures must be used if sperm numbers are limited or sperm quality is poor. Classic methods for in vitro fertilization, in which the sperm and oocyte are co-incubated, have had limited success in the horse. Oviductal insemination with placement of a limited number of sperm and the oocyte into the oviduct have been tried in a process called gamete intrafallopian transfer (GIFT). Under experimental conditions, GIFT was successfully used to produced pregnancies, using 2×10^5 fresh sperm that had not been exposed to a semen extender and when transferred with oocytes into recipients' oviducts.[13] Although 82% of oocytes were fertilized when fresh sperm were used, fertilization rates were significantly lower for cooled and frozen sperm (25 and 8%, respectively).[13] Therefore, although procedures for GIFT are feasible in a clinical settings, the semen requirements can make the procedure difficult.

Currently, the most reliable method of assisted fertilization in vitro is through the technique of intracytoplasmic sperm injection (ICSI). The procedure involves the selection and injection of a sperm into the ooplasm of a mature oocyte. Use of ICSI provides a sperm efficient method of fertilization, allowing offspring to be produced with limited quantities of sperm or poor quality semen. Typically, sperm used for ICSI are frozen and thawed, but the procedure can be done with cooled or fresh semen. Immature[6,14] and maturing[12,15] oocytes have been collected from donors and used to produce pregnancies for clinical cases with subfertile mares or limited or poor-quality semen.

Fertilized oocytes usually cleave into two cells within a day of the sperm injections. The early embryos can be transferred into the oviduct of a synchronized, cyclic recipient by standing flank laparotomy. Alternately, embryos can also be cultured in vitro until the morula or blastocyst stage of development; at this time, they can be transferred into recipients' uteri. Embryos remaining in culture too long often try to unsuccessfully "hatch" from the zona pellucida. In vivo, the zona pellucida will thin as the blastocyst expands and an acellular layer, called the capsule, is formed between the trophoblast and zona pellucida. Under current culture conditions, the capsule is not formed, and the zona pellucida does

not fully expand. Therefore, embryo should be transferred into a recipients' uterus before the trophoblast tries to extrude from the zona pellucida. The embryos can be transferred using standard procedures for transcervical embryo transfers, with the recipient synchronized to be four to six d after ovulation. Cells in the ICSI-produced embryos usually cleave at 12- to 24-h intervals, with formation of a blastocyst at approximately six d. However, some ICSI-produced embryos in our laboratory have been delayed by days in development and still resulted into viable pregnancies.

The ICSI procedure is difficult in most clinical situations. The micromanipulation and incubation equipment is expensive and requires clean conditions, a controlled environment, diligent monitoring, and regular upkeep. Specific expertise is required to conduct the procedure and maintain quality control. However, some options exist for the equine practitioner to incorporate the procedure into their practice without investing in the equipment and expertise for ICSI. Oocytes can be collected at one facility and shipped to a central facility for ICSI and embryo culture. When shipping maturing oocytes, we instruct referring veterinarians to ship them in a portable incubator maintained at approximately body temperature. The oocytes can be collected in the afternoon at approximately 20 to 24 h after hCG or deslorelin and shipped by ground parcel overnight. Upon arriving at their destination, ICSI can be performed. More flexibility may be possible for immature oocytes.

Once an embryo is produced, it can be transferred at the central facility, cryopreserved[7,16] or shipped back to the facility of origin. We have shipped ICSI-produced embryos in cooled containers at approximately 5°C or in portable incubators set at 37°C.

Harvesting of oocytes

The equine practitioner can be tasked with having to euthanize a mare because of a medical condition. In an attempt to produce additional offspring, the ovaries can be harvested, and the remaining gametes collected. Equine practices intending to offer these services to clients should be prepared for ovarian removal and shipment[6,17] and with contact information of referral facilities. The practitioner can make a general assessment of potential success based on ovarian activity and other factors associated with the mare. The ovaries should have some follicular activity. Immature fillies or old mares, mares under protracted stress, pain or medical treatments, and anestrous mares often do not provide good quality oocytes. When possible, the referral facility should be contacted prior to euthanasia for recipient preparation, timing of procedures, and shipping instructions. Ovaries are usually removed directly after euthanasia; however, if the potential occurs to obtain the gonads prior to euthanasia, for example during colic surgery, it could be beneficial to remove the ovaries prior to the administration of the euthanasia solution. After euthanasia, the easiest method to remove the ovaries is often to make an incision in the flank area, locate the ovary, and cut the surrounding ligaments and connections. The second ovary can usually be identified and removed through the same incision. Alternately, a second incision can be made in the opposite flank or a midline incision can be used. The ovaries should be kept as clean as possible to avoid contamination of the tissue and gametes. Upon collection, the ovaries should be rinsed in a physiological salt solution, such as an embryo collection medium. The ovaries are then placed in clean, plastic bags with a small amount of medium and secured.

If the interval to the laboratory is less than one h, a temperature of 37 to 38°C is preferred; if a longer shipment is required, the shipment temperature should be lowered to cool room temperature or approximately 20°C.[6,17] The ovaries should not be held at 5°C or colder for any length of time. In our laboratory, offspring have been produced from ovaries reaching the facility after almost one d after removal. However, the shortest possible interval of transport is preferred. In some cases, shipment by overnight ground courier is more economical and reliable than connecting air flights.

Alternately, oocytes can be collected at the facility of origin and shipped in culture medium in a portable incubator or at room temperature.[6] For oocytes to be collected at the facility of origin, proper techniques for oocyte collection and handling are needed. The firm attachment of the cumulus oocyte complex to the follicular wall prevents the oocyte from being easily aspirated from follicles, and the follicles are often incised and scraped using a bone curette.[6,17] Oocytes must be placed in an appropriate

medium for shipment, which can usually be obtained from the referral facility if sufficient time is available.

Harvested oocytes are usually cultured for 24 to 30 h for in vitro maturation prior to oocyte transfer or ICSI. Equine oocytes have been successfully cryopreserved, warmed and resulted in viable foals;[18] but the efficiency is not high. Therefore, harvested oocytes are usually transferred, or ICSI-produced embryos are shipped to another facility or cryopreserved if appropriate recipients are not available. The first clinical offspring from harvested, shipped ovaries was born in 2002,[19] and more offspring have been successfully produced from some referral facilities,[1,6] with oocytes or ICSI-produced embryos transferred at the facility or after shipment to another location.

Summary

As advances are made in ART, the equine practitioner has new opportunities to promote new clinical procedures or provide referral consultations. Establishing a facility for some ART procedures, such as ICSI, is not feasible for many clinics. However, establishing methods to collect and ship oocytes or transfer ICSI-produced embryos can be incorporated. Services, such as harvesting and shipping ovaries or oocytes, can be relatively easily incorporated into most equine practices.

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Incorporating non-antibiotic anti-infective agents into the treatment of equine endometritis

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Abstract

Chronic endometritis remains an important cause of fertility in the mare. Conventional therapy (antibiotics and antifungals) aimed at resolving chronic endometritis sometimes fails due to a variety of reasons including uncorrected anatomic abnormalities and the presence of abnormal mucus or biofilm in the uterus. With increasing frequency, bacteria with multiple resistance patterns are being recovered from the equine uterus. Therefore, alternative methods to treat microbial infections are needed to reduce the reliance on traditional antimicrobials. The rationale, preparation, and protocols for use of non-antibiotic anti-infective agents including mucoactive agents (N-acetylcysteine), buffered chelators (EDTA-Tris and Tricide™), non-classified solutions (dimethyl sulfoxide, dilute vinegar and povidone-iodine solutions, hydrogen peroxide) and immunomodulatory agents (glucocorticoids, cell-wall extract of *Mycobacterium phlei*, *Proprionibacterium acnes*) are discussed.

Keywords: Equine, endometritis, non-antibiotic agents, mucoactive agents, buffered chelators,

Introduction

Despite the elucidation of uterine clearance mechanisms in the mare and substantial progress in identifying effective treatment for persistent mating-induced endometritis, chronic endometritis remains an important cause of infertility. Conventional therapy (antibiotics and antifungals) aimed at resolving chronic endometritis sometimes fail due to a variety of reasons including uncorrected anatomic abnormalities and the presence of abnormal mucus or biofilm in the uterus. Bacterial biofilms are complex populations of multiple microbial species embedded within a glycocalyx matrix,[1] which can confer up to a 500-fold increase in bacterial resistance to antibiotics compared to traditional in vitro pure culture.[2] With increasing frequency, bacteria with multiple resistance patterns are being recovered from the equine uterus. These observations highlight the need to find alternative methods to treat microbial infections rather than to continue to rely solely on traditional antimicrobials. The following will review the rationale and use of non-antibiotic anti-infective agents: mucoactive agents, buffered chelators, non-classified solutions, and immunomodulatory agents.

Mucoactive agents

Certain mucus hypersecretory disease processes, such as chronic obstructive pulmonary disease (COPD), asthma, and cystic fibrosis, are characterized by an overproduction of mucus which overwhelms the ability of ciliated epithelial cells to move the mucus to the pharynx for swallowing. Mucoactive agents affect either the amount (mucoregulators), viscosity (expectorants, mucolytics), or clearance of mucus (mucokinetics), with the end goal of improving the efficiency of the mucocilliary apparatus (for review see[3]). Several agents that have been used for the treatment of chronic endometritis are potentially mucoactive and may have a beneficial effect on disrupting abnormal mucus on the surface of the equine endometrium.[4] Although the mucoactive properties of these agents in the equine uterus have not been definitively identified, glucocorticoids (mucoregulator) and N-Acetylcysteine (mucolytic) show promise in this regard.

N-acetylcysteine (NAC) is cysteine modified by the addition of an acetyl group to the nitrogen atom. The sulfhydryl group confers antioxidant properties,[5,6] and can also reduce the disulfide bonds in mucin, thereby decreasing the viscosity of mucus.[7] For intrauterine therapy in the mare a 3.3% solution is prepared by diluting 30 mL of a 20% solution of NAC to 150 mL of sterile saline. In a recent safety study, mares received either NAC or saline on day 1, followed by uterine lavage on days 2 and 3, and endometrial biopsy on day 4. No detrimental effects on the endometrium were observed, and the thickness and staining intensity of extracellular mucus was reduced in NAC-treated reproductively

healthy mares. In the accompanying clinical trial, first cycle pregnancy rates for repeat-breeder mares with abnormal mucus receiving NAC 24 to 36 h prior to mating, mares with negative culture the cycle prior to mating and no pre-mating treatment, and mares treated with antibiotics the cycle before mating and no NAC pre-breeding were 77%, 74%, and 56%, respectively. Although these rates were not statistically significant, there was no difference in the first cycle pregnancy rates of the repeat breeder mares and those not having endometritis on the cycle prior to mating (healthy mares). Interestingly, healthy mares receiving uterine lavage, antibiotics and ecbolics post-mating had significantly lower pregnancy rates (62%) than those receiving uterine lavage and ecbolics alone (89%). The authors speculate that a decrease in mucus viscosity induced by NAC-induced facilitates sperm transport.[8]

Buffered chelators

Buffered chelators have been shown to potentiate antimicrobial agents in a variety of settings,[9,10] presumably by altering cell wall integrity following removal of divalent cations from the outer bacterial membrane or cell wall.[11] The first reported use of an early generation buffered chelator (ethylenediaminetetraacetic acid-2-amino-2-hydroxymethyl-propane-1,3-diol; EDTA;-Tris) caused no deleterious effect on the endometrium[12] and in vitro reduced the MIC of a strain of *Pseudomonas aeurginosa* recovered from cases of endometritis.[13] In the cow, a comination of antibiotics with EDTA-Tris infusion was more effective than antibiotics alone in resolving bacterial endometritis.[14] More recently a third-generation buffered chelator (disodium ethylenediaminetetraacetate dehydrate-2-amino-2-hydroxymetheyl-1,3-propanediol (Tricide™, Molecular Therapeutics, LLC, Athens, GA) has been shown in vitro to potentiate the antimicrobial effect of antifungal agents examining isolates obtained from clinical equine fungal keratitis cases.[9] Using *Escherichia coli* as a model, the mode of action of EDTA on cell viability is through alteration of the cell wall permeability[15] and structural integrity.[16] For equine endometritis, the recommended therapeutic protocol is to lavage the uterus with lactated Ringer's solution (LRS), instill the chelator for 12 to 24 h (250 to 1000 mL), and lavage the uterus on subsequent days to remove potential exudates and debris. No ecbolics are given at the time of chelator infusion and the volume of chelator is based on the size and positioning of the uterus. Appropriate antimicrobial agents may be directly mixed (amikacin, ampicillin, fluroquinolones, clotrimazole, and fluconazole) with the chelator solution. Treatment with the chelator can be repeated during the same estrus if deemed appropriate, and treatment and mating during the same estrus can result in pregnancy.[17]

Other anti-infective agents

Dimethyl sulfoxide ($[CH_3]_2SO$; DMSO) is an amphipathic molecule with a highly polar domain and two apolar domains, allowing its solubility in water and organic matter and making it an excellent solvent. In addition to its solvent properties, DMSO is an effective anti-inflammatory agent and reactive oxygen scavenger (ROS), and has been used for a variety of diseases such as interstitial cystitis, amyloidosis, colitis, pancreatitis, and prostatitis (for review see[18]). In the mare, DMSO was initially examined for its potential effects on reducing endometrial periglandular fibrosis through anti-inflammatory and fibrinolytic effects. Published protocols were 100 mL 75% DMSO on day 1, followed by five days of 100 mL 25% DMSO;[19] and 60 mL of 10, 20, or 30% DMSO for five days.[20] Results varied from no having no benefit[19] to a reduction in fibrosis.[20] Treatment with 30% DMSO for five days also produced a significant improvement in the chronic inflammatory infiltrate of barren mares compared to control mares (66% and 11%, respectively), and a higher but non-significant pregnancy rate in treated mares (76%) compared to controls (53%).[20] The effect of DMSO on the secretory activity of goblet cells and the height of the mucus blanket were not variables in either study, so it remains a possibility that improvement in fertility was through a reduction in mucus hypersecretion. Recently, *Pseudomonas* Flagellin/TLR5 stimulation of epiderminal growth factor receptor and mucin overproduction in human bronchial epithelial cells was inhibited by the ROS activity of DMSO.[21] In addition to beneficial effects on the host, DMSO displays direct antimicrobial properties as well. At a 30% concentration, DMSO was bactericidal for *Escherichia coli*, *Proteus vulgaris*, Group A ß-hemolytic streptococci, and *Candida albicans*, while a 10% solution was bactericidal for *Pseudomonas aeruginosa*; 40% DMSO was required

for bactericidal activity against *Staphylococcus aureus*. Lower concentrations (5 to 10%) were generally bacteriostatic, and there was no potentiating effect of DMSO for antibiotic agents at any concentration tested.[22] Commercial preparations of DMSO range from 90 to 99%. For intrauterine use, DMSO is typically infused as a 10 to 30% solution, and can be used daily. In order to prepare a 30% solution using 90% DMSO, 33 mL of a 90% DMSO solution is added to 64 mL of sterile saline.

Distilled (white) vinegar is acetic acid and water, typically at concentrations ranging from 5 to 8% acetic acid, and having a pH of 2.4. The rationale for the use of acetic acid in the treatment of endometrial fungal infections likely stems from its use for human otitis externa, onchomycosis, and bacterial vaginosis. A recent systematic review of the literature on otitis externa therapy concluded that conventional topical antibiotic therapy lacks efficacy, symptoms frequently recur, and that dilute vinegar solution is a viable alternative to conventional antibiotics.[23] For intrauterine use a 2% solution (10 mL vinegar in 450 mL saline) is infused, left in for five min, and then completely removed by large-volume lavage (2 L). Since the goal of treatment is a solution with a low pH, sterile saline is a more logical diluent than is LRS. It is important to completely remove the dilute acetic acid solution by lavage; failure to do so usually results in severe irritation to the reproductive tract. An appropriate antimycotic agent can then be instilled immediately following the lavage.

Dilute povidone-iodine solution is advocated as an antiseptic for mares with fungal endometritis, and is prepared as a 0.01 to 0.05 % (actual percentage of iodine) solution by diluting 1 to 5 mL of a 10% povidone-iodine solution (1% available iodine) in 1000 mL of sterile saline, and then used as a lavage fluid. Uterine lavage with a 0.05% povidone-iodine solution four h after mating had no detrimental effect on fertility.[24] Some mares are highly reactive to povidone-iodine and can develop a rather marked cervicitis and vaginitis if the solution is not completely removed from the uterine lumen; lavage of the uterus with sterile saline or LRS following irrigation with the dilute iodine solution is recommended.[25] Povidone-iodine solutions vary in the percentage of iodine between 7.5 and 12%, so checking the content of the solution is advised.

Hydrogen peroxide (H_2O_2) as a 1% solution was successful in resolving recurrent bacterial vaginosis in women resistant to other forms of treatment,[26] and was effective in reducing clinical symptoms of endometritis in early post-partum cows, and reducing bacterial contamination but this latter effect was not significant.[27] A 1% solution is prepared by diluting 20 mL of a 3% solution of hydrogen peroxide in 60 mL of LRS. Infusion should only be performed during estrus.[28]

Autologous plasma was advocated in the 1980s and early 1990s as a method to supplement the intrauterine environment with complement and immunoglobulins, both of which are key components for bacterial opsonization,[29] but its use declined as information on the importance of mechanical clearance mechanisms came to the forefront. In a large field study of 1341 breeding cycles, the addition of autologous plasma to a post-breeding infusion of antibiotics significantly improved pregnancy rate per cycle in lactating mares, tended to improve pregnancy rate per cycle in barren mares, and had no effect on maiden mares.[30] To prepare, one L of venous blood is collected asceptically into a receptacle containing 10,000 IU heparin. While gravity separation under refrigeration is acceptable, centrifugation under refrigeration is preferred. Complement components are very labile, and are best preserved by prompt separation and immediate freezing. Plasma is aliquoted into 100-mL doses and can be stored at -20°C for short periods of time (weeks). If the plasma is to be held for longer periods of time, storage at -70°C is required to preserve complement activity.

Chemical curettage with commercial kerosene was first advocated by Charles Roberts of New Zealand. A single 50-mL infusion of kerosene produced glandular activation in all mares, while inflammation took longer to resolve (14-21 d) in Grade III mares compared to Grade I and II mares (4-7 d). Five of ten mated Grade II mares conceived and carried to term, while nine of 11 mated Grade III mares conceived and five carried to term.[31] Kerosene remains popular to some degree in certain geographic regions, but is generally considered a last resort. Its success is theorized to result from sloughing of the luminal epithelium, especially the goblet cells, with a return to a more normalized state of the mucus blanket.[32] Treatment can be during diestrus; if in estrus, digitally occlude the cervix for a short period of time to prevent vaginal reflux.[33]

Immunomodulatory agents

Therapy aimed solely at aiding physical clearance can be associated with treatment failures, which has stimulated interest in modulation of the inflammatory response. A significant improvement in pregnancy rate was observed when prednisolone acetate (0.1 mg/kg, q 12 h) was administered to mares with a history of post-mating-induced endometritis at the same time as administration of human chorionic gonadotropin, and then continued until detection of ovulation.[34] Results with dexamethasone have differed depending on the dose and time of administration relative to insemination. When dexamethasone (50 mg, i.v.) was administered within one hour of breeding in combination with traditional post-breeding therapies (ecbolics, uterine lavage) to mares with a history of fluid accumulation, an increase in pregnancy rate was observed if the mare was affected with three or more risk factors for susceptibility to endometritis. Risk factors identified were abnormal history, positive culture, ≥2 cm fluid pre-breeding, poor perineal conformation, abnormal cervix, post-breeding fluid >1.5 cm and <2 cm, post-breeding fluid ≥2 cm, no post-foaling repair of a previous vulvoplasty, reproductive tract abnormalities, and fluid present 36 h post-breeding.[35] However, when dexamethasone (10 or 20 mg, i.v.) was administered six to 12 h after insemination in combination with additional treatments to mares with a history of fluid accumulation after breeding no increase in pregnancy rates was observed.[36] These conflicting findings highlight that dose and timing of treatment, and risk factors for endometritis play important roles in the response to immunomodulation with steroid treatment. Completely separate from the proposed effect of glucocorticoids on modulating the immune response is the potential that glucocorticoids may provide beneficial regulation of mucus production.[3] Enhancement of cell-mediated immunity by a cell-wall extract of *Mycobacterium phlei* (Settle®; Bioniche Animal Health, Bogard, GA) resulted in a more rapid clearance of inflammation in mares with experimentally induced *Streptococcus equi* subspecies *zooepidemicus* infection.[37] Co-administration of traditional therapies and *Proprionibacterium acnes* (EqStim®; Neogen Corp, Lexington, KY) to barren mares with persistent endometritis was associated with greater pregnancy and live foal rates than compared to traditional therapies alone.[38]

Conclusions

From the foregoing discussion is it apparent that there are options for incorporating non-antibiotic or non-antifungal agents into treatment protocols for chronic endometritis, with the potential for overcoming bacterial and fungal resistance. For a summary of the different indications, preparation, and protocols for the use of non-infective agents the reader is directed to the Table. Unfortunately treatment failures and relapses may still occur, but utilization of these agents in conjunction with traditional therapies will, in general, reduce the rate of failure and the number of matings to conception.

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Table: Indication, preparation, and protocol for nonantibiotic antiinfective agents for intrauterine use in the mare.

Antiinfective agent	Indications	Preparation	Use	Comments /Reference
N-acetylcysteine (NAC)	Repeat breeders; abnormal mucus	30 mL of 20% NAC in 150 mL sterile saline	Infuse 24-36 h pre-mating; post-mating lavage + ecobolics	(32)
Buffered Chelator (Tricide™)	Chronic gram negative endometritis, fungal endometritis, antibiotic resistance	Compounding pharmacies; or 20-gm packet in 3.78 L sterile water over heat + stirring, aliquot in 500 mL, store in dark room temp	Lavage, then Infuse 250-1000 mL leave in, no ecobolics Lavage on subsequent days; can repeat on same estrus	(39) Can mix some antimicrobials in chelator solution; can be used on same cycle as mated
DMSO	Chronic endometritis; abnormal mucus	30% soln; 33 mL (90% DMSO) + 64 mL saline	Infusion daily up to 5 days during estrus	(20)
Vinegar	Fungal endometritis	10 mL vinegar + 450 mL saline	Infuse 500 mL leave in 5 min; Lavage 1-2 L	Can repeat in same estrus
Povidone-iodine	Fungal endometritis; chronic endometritis	1 to 5 mL of a 10% povidone-iodine solution in 1000 mL saline	Used as lavage solution (1 L); advised to follow lavage with LRS until clear	(24,33)
Hydrogen peroxide	Chronic endometritis	20 mL of 3% H2O2 in 60 mL LRS	Infusion	(28) Only during estrus

Techniques to enhance the use of suboptimal semen - a practitioner's approach

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Abstract

The clinical use of semen processing techniques, including cushioned centrifugation and density gradient centrifugation, is discussed. Case scenarios involving these techniques, coupled with fertility trials to determine lowest effective insemination doses, are used to illustrate how semen processing can increase breeding efficiency (pregnancy rates achieved per cycle or season; number of pregnancies established) for subfertile stallions with high demand.

Keywords: Stallion, semen quality, cushioned centrifugation, density-gradient centrifugation, low dose insemination

Introduction

Stallions are often offered to the public to breed mares without regard to their potential fertility. This has led to demand for breeding of a significant number of stallions that, while they may produce desirable performance traits in progeny, are subfertile (for a variety of reasons). Semen characteristics may vary greatly among stallions in a given breed, particularly amongst those that are subfertile.[1] Nevertheless, suboptimal semen quality and quantity is common in immature stallions recently retiring from performance careers,[2] as well as in aging stallions suffering from testicular dysfunction.[3] There are many mature stallions that have poor semen quality of undetermined cause.[4] Due to the financial incentive to book as many mares as possible to a stallion, owner/manager expectations for the number of mares bred per year often exceeds the stallion's spermatogenic potential. The common use of cooled transported and frozen semen by many breeds, compounded by an oftentimes casual approach to semen processing by many suppliers, can further hamper the ability to produce pregnancies in an efficient manner. Consequently, the equine breeding industry abounds with stallions that actually achieve less-than-desirable pregnancy rates.

Thus, there is a need for veterinary practitioners to assist stallion owners/managers to maximize pregnancies achieved by subfertile stallions. In our practice area, this demand often occurs only after routine breeding management procedures have failed. This communication discusses the clinical use of previously described techniques involving advancements in centrifugation of semen, often incorporated with deep-horn low-dose insemination techniques, to enhance pregnancy rates or the number of pregnancies produced by stallions used in artificial insemination programs. Our primary goal has been to improve the per-cycle pregnancy rate of a given stallion, while concurrently inseminating more mares per ejaculate. Subsequently, we have been able to generate income for the veterinary practice, while simultaneously producing a significant return on investment to stallion management, by securing more breeding fees and increasing the number of pregnancies produced by a given stallion.

Stallion evaluation

The plan to maximize fertility of a given stallion begins with an evaluation of the complete breeding process, in addition to a thorough breeding soundness examination.[5] The entire semen-collection and preparation processes should be evaluated, including cleaning and storage of reusable labware, preparation of the artificial vagina, method of sperm concentration determination, semen volume estimation, and motility estimations.[1] Fertile horses that only breed a small numbers of mares may tolerate unfavorable laboratory techniques or semen processing, while less fertile stallions in high demand will not.

Breeding history is invaluable, but is sometimes difficult to obtain.[6] Per-cycle and seasonal pregnancy rates for prior seasons and pregnancy rates to-date in the current season should be calculated. Pregnancy rates for different classes of mares (e.g., maiden, lactating, and barren mares) are helpful, if available. Numbers of sperm per inseminate and pregnancy outcomes for breedings with fresh semen

used on-site and for transported semen used off-site should be compared to provide historical data for later comparison to pregnancy rates achieved following any subsequently instituted changes in semen processing, number of sperm inseminated, or method of insemination.

A complete breeding soundness examination[5] should be performed to generate data to identify areas where altering breeding management strategies could possibly improve pregnancy rates. Predicted daily sperm output (DSO) determined from total testicular volume measurements[7] are compared to actual total sperm in the ejaculate (at DSO) to estimate spermatogenic efficiency.[8] Awareness of total sperm in the ejaculate at DSO, and typical volumes and concentrations for a complete ejaculate, of the given stallion is imperative for allowing judgments to be made regarding potential semen processing techniques that could be practical for use. Obtaining and processing multiple ejaculates are often required to allow such determinations to be made. Morphologic profiles determine the relative percentage of normal sperm to abnormal sperm and identify specific abnormalities that might be contributing to suboptimal fertility. Sperm chromatin quality of fresh raw semen is determined by the sperm chromatin structure assay.[9,10] Percentage of total and progressive sperm motility in different extenders are determined for fresh semen and for extended-cooled semen following 24 and 48 hours of storage. These assessments provide baseline values of semen quality for comparison to those obtained by varying semen processing techniques that are used.

Proficient semen collection technique

While a "how to" on stallion collection is beyond the scope of this presentation, it is the author's view that regardless of techniques used to process semen to potentially enhance fertility, the success of said efforts is dependent, to a large degree, on the collection process itself. All ejaculates are not equal. The volume of pre-ejaculatory secretions from the bulbourethral glands, secretions from the accessory sex glands (particularly gel from the seminal vesicles) and relative sperm concentration affect semen viscosity and therefore the sperm sedimentation rates that are achieved during centrifugation. Water soluble lubricant and other contaminants present in the ejaculate can alter the osmolarity and pH to a degree that adversely impacts sperm quality. The goal should be to obtain a complete clean ejaculate in a single mount with minimal sexual stimulation of the stallion before collection, all in order to obtain an ejaculate with relatively low volume and high sperm concentration. The importance of a skilled stallion handler and basic ground training of the stallion cannot be over emphasized in this regard.

Cushioned centrifugation

Centrifugation of semen is oftentimes indicated to concentrate dilute ejaculates and remove excess seminal plasma. Ideally, centrifugation should result in a 100% sperm recovery rate with no resulting damage to sperm quality. Loss of a significant portion of the ejaculate, leaving fewer sperm available for breeding purposes, could negate the benefit of concentrating the insemination dose. The main concern when attempting to maximize sperm recovery through centrifugation is the adverse effect that centrifugation can have on the integrity of sperm. Typically, an increase in centrifugation time or gravitational (*g*) force results in an increased sperm recovery rate, but it can also lead to decreased sperm motility or quality because of the mechanical forces associated with centrifugation and excessive packing of the sperm.[11-13]

Recently, a cushioned centrifugation procedure has been applied to stallion semen to maximize sperm harvest without attendant injury to sperm. A non-ionic iodinated compound, iodixanol, was first reported for density-gradient cell fractionation and has since been used as either a density gradient or a cushion for centrifugation of sperm.[14-21] Investigations regarding cushioned centrifugation of stallion semen with this product showed excellent yields of sperm that were undamaged by the centrifugation process, but an optically clear centrifugation medium was required to reduce sperm losses. Texas workers found that cushioned centrifugation of stallion semen in either 50 mL conical- bottom tubes containing 3.5 mL of iodixanol solution and centrifuged for 20 min at 1000 x *g*, or 45 mL nipple-bottom tubes containing 30 µL of iodixanol solution and centrifuged for 20 min at 400 x *g*, yielded a high sperm harvest while maintaining sperm function.[22] They also noted an optically opaque extender, as is typically

used in the equine breeding industry, was suitable to achieve this goal. The nipple-bottom tubes are recommended over the 50-mL conical-bottom tubes for cushioned centrifugation when the sperm number in ejaculates is relatively low (i.e., less than 2–3 x 10^9 sperm) or when it is necessary to remove more seminal plasma from sperm after centrifugation than is possible with cushioned centrifugation in conical-bottom tubes. Recently, it has been demonstrated that the volume of iodixanol solution can be reduced from 3.5 to 1 mL in 50 mL conical-bottom tubes without impairing sperm harvest or semen quality.[23]

Three proprietary iodixanol products are available: 1) Otiprep™ (Sigma-Aldrich, St. Louis, MO; #D1556- 250 mL, hypotonic, 170 mOsm/L); 2) Cushion Fluid™ (Minitüb, Tiefenbach, Germany; isotonic, 300 mOsm/L); and 3) Maxifreeze™ (IMV, L'Aigle, France; isotonic, 300 mOsm/L). The iodixanol products cushion the sperm during centrifugation to allow concentration of sperm for preparing deep horn insemination doses, prior to mixing semen with freezing extenders for cryopreservation, prior to final extension for cooled storage, prior to placing a small volume over density gradients, and perhaps to aid in removal of urine and urinary sediment when ejaculates are contaminated with urine.

Density-gradient centrifugation

Discontinuous density centrifugation to aid in separation of better-quality sperm has enjoyed broad clinical application in recent years. Density-gradient centrifugation is relatively simple to perform, and has been shown to effectively separate sperm with various morphological features in an ejaculate.

Centrifugation of equine semen through a silanated silica-particle solution (EquiPure™, Nidacon International AB, Mólndal, Sweden) has shown promise for selecting sperm with good motility, morphology, and chromatin quality[24,25] and enhancing the fertility of selected subfertile stallions.[8] Sperm recovery rate has been found to be higher when 2-4 mL of Equi Pure™ Bottom Layer is used in 15-mL capacity conical-bottom tubes[25] than with double layer (Equi Pure™ Top Layer plus Equi Pure™ Bottom Layer). Centrifugation of semen through a silica-particle solution, such as EquiPure™, is not a logical approach for stallions with normal semen quality, because a relatively high percentage of the sperm population can be lost after centrifugation. Because this technique results in sperm separation based on sperm buoyancy or isopycnic point, its use is most justified when an ejaculate contains a high percentage of sperm with morphologic defects, particularly sperm with abnormal heads, abnormal midpieces, bent midpieces, bent tails, coiled tails, or premature (round) germ cells. However, it has been found that the technique sometimes improves chromatin quality in the recovered sperm population, regardless of sperm morphologic profile. The technique can also be used when more complete separation of seminal plasma from sperm is desired.[26]

Low dose insemination

The minimum effective number of sperm that can be used to inseminate mares to achieve commercially acceptable pregnancy rates is a subject of much debate in recent years.[27] A single ideal number that can be applied in all cases eludes us. A more rational scenario would seem to indicate that the appropriate threshold number of sperm is related to the fertility of a given stallion and the insemination procedure that is applied (e.g., fresh or cooled-transported semen). Industry standards have typically been to inseminate 200-500 x 10^6 progressively motile sperm into the uterine body when fresh semen is used, or typically beginning with 1 x 10^9 progressively motile sperm prior to cooling when semen is to be chilled and transported.[27] However, a recent report revealed no difference in pregnancy rates when 50 to 300 million fresh sperm from normal fertile stallions was used to inseminate reproductively normal mares.[28]

Rigby et al[29] demonstrated that only 0.0007% of sperm that are deposited into the body of mare uteri actually gained access into the oviducts to be available for fertilization of an oocyte. They found that insemination in the tip of the uterine horn ipsilateral to an ovary containing a dominant follicle resulted in a greater percentage of oviductal sperm (77%) occupying that oviduct than with insemination in the uterine-body (54%). They concluded that more sperm gain access into the desired oviduct following deep-horn, compared to uterine-body, insemination.

This technique for low-dose insemination has been investigated in the research setting and applied clinically in recent years. Two techniques are commonly used to accomplish deposition of an insemination dose on, or near, the oviductal papilla: (1) use of an endoscope to visually confirm placement of semen on the papilla through a long catheter passed through the biopsy channel, and (2) use of a flexible pipette in which the tip is guided to a position near the oviductal papilla by manipulation per rectum, before deposition of the insemination dose. The optimal method for insemination of very low doses of sperm is a subject of some controversy.[30-32] Hayden et al found no significant difference in pregnancy rates achieved between the two procedures when mares were inseminated with sperm numbers below the determined threshold required to achieve normal pregnancy rates (i.e., 0.5-1 x 10^6 sperm from a stallion with known good fertility).[33]

The value of low-dose insemination for improving fertility of subfertile stallions has been questioned, although apparently successful results with this breeding strategy exist.[8,26,34] Contributing further to the controversy is whether low dose insemination using separated sperm (e.g., density gradient centrifugation, etc.) results in more normal sperm actually colonizing the uterotubal junction (UTJ) and oviduct, and actually improves fertility. Non-proponents for breeding with separated sperm argue that natural selection for normal sperm at the UTJ/oviduct precludes most abnormal sperm from participating in the fertilization process. Certainly, a much higher percentage of those sperm that actually colonize the UTJ are morphologically normal than are present in the entire inseminate.[35,36] Proponents for breeding with separated sperm suggest that fewer uncompensable sperm defects will be present that might access the oviduct and lead to fertilization failure or early embryonic death. Obviously, more research in this area needs to be done. However, this author is convinced that the low dose insemination strategy is a valuable tool when combined with an appropriate centrifugation procedure for preparation of the insemination dose.

Case 1

A 12-yr old Quarter Horse stallion was presented on April 1, 2003, with a history of impregnating 35 of 100 mares bred with fresh semen on the farm. A review of breeding records revealed 14 pregnancies from 45 cycles of breeding in the month of March (31% pregnancy rate per cycle). Mares had been bred with at least a "shipping dose"(1 x10^9 progressively motile sperm) based on sperm concentrations obtained with a densimeter (Animal Reproduction Systems, Chino, CA) and visual estimation of sperm motility (estimated typically to be approximately 60%). Mares bred per ejaculate ranged from one to five, most often being three or four. Total estimated number of sperm per insemination dose used ranged from 2-8 x 10^9.

Total testicular volume of this stallion was determined to be 308 cc, resulting in a predicted DSO of 6.1 x 10^9, assuming that the testes were producing sperm with normal efficiency. The stallion had been bred 35 times in the last 60 days (approximately every other day), and total sperm number obtained in an ejaculate was 13.87 x 10^9; therefore, spermatogenic efficiency was considered to be normal. Sperm motility was estimated to be 50%/35% (total/progressive), with velocity estimated to be 3 of 5 (moderate). The percentage of morphologically normal sperm in the ejaculate was 18%. The most common morphological defects were abnormal heads (39%), abnormal midpieces (22%), bent tails (32%) and coiled tails 4%. The sperm chromatin structure assay yielded 30%COMP-αt compared to 6% for semen from a control stallion of known good fertility, indicating a high percentage of sperm had unstable DNA.

With the morphological abnormalities and the degree of abnormal DNA present in the stallion's sperm, it was unclear whether increasing the numbers of sperm inseminated would improve pregnancy rates. We reduced the number of mares bred per ejaculate to two, and achieved nine pregnancies from 14 cycles in seven mares (64% pregnancy rate per cycle). While it appeared that increasing sperm numbers per inseminate would improve fertility, the mare book would have to be reduced for the rest of the season.

Pregnancy rates improved as increased numbers of sperm were inseminated into the uterine body, so the plan was to breed more mares per ejaculate by using deep-horn insemination to see if placing the sperm as near to the oviductal papilla as possible would still produce acceptable pregnancy rates with a

reduced (compared to uterine body inseminations) number of sperm. Management was changed so that four or five mares were bred per ejaculate, which resulted in insemination doses ranging from 1.28-2.6 x 10^9 in 10-20 mL extended semen, using a transrectally-guided deep horn insemination technique. By utilizing this technique, an additional 52 pregnancies were obtained from 117 cycles by July 10. End-of-season (total of all methods of breeding throughout the year) results for this stallion were: pregnancy rate per cycle 43.6% (106/243); seasonal pregnancy rate 96% (106/110).

The same breeding protocol was implemented from the beginning of the next (2004) breeding season, with an addition of a change in mare management. Mares were palpated daily and an ovulation-inducing agent was administered the day prior to breeding, to ensure most mares would be bred within 24 hours prior to ovulation. Pregnancy rate per cycle for mares ovulating within 24 hours after breeding was 61%, while pregnancy rate per cycle for mares ovulating between 24 and 48 hours after breeding was 44%. Overall, a 54% pregnancy rate per cycle was achieved, resulting in a seasonal pregnancy rate of 96% (115/120) by May 15 rather than July 10, despite no apparent differences in sperm output, morphological profile, or sperm chromatin quality in the stallion's ejaculates.

The same management strategy produced similar results through mid-March of 2005 when a precipitous decline in pregnancy rate per cycle occurred (i.e., from 69% achieved during the first six weeks of the season to 39.4% (28/71) achieved from mid-March through the end of April). No obvious difference was detected in sperm motility estimates during this decline in fertility, but the percentage of morphologically normal sperm declined to 10% due primarily to an increase in abnormal heads. Sperm chromatin structure assay values were greatly elevated, demonstrating marked sperm DNA instability consistent with that of stallions that are extremely subfertile. Subsequent pregnancies were expected only to occur as random events unaffected by sperm numbers or insemination technique. The decision was made to try concentrating the semen using cushioned centrifugation, followed by deep-horn insemination. The ejaculates were concentrated and divided between two to five mares with 2-6 x 10^9 sperm in 5-8 mL volumes and mares were inseminated with a transrectally-guided deep horn technique. Pregnancy rate per cycle returned to 58% for the remainder of the season, resulting in an overall 92.4% (105/114) seasonal pregnancy rate.

Case 2

A 4-yr-old Quarter Horse stallion was presented to manage for breeding following an evaluation at the Texas Veterinary Medical Center for breeding soundness. The stallion had concluded his first season at stud where he achieved a 59% seasonal pregnancy rate when covering 165 mares. Approximately one-half of the mares had been bred with cool-transported semen. During that first season, progressive sperm motility was estimated to be 70% for 76 of 84 semen collections performed, with an average total sperm number in the 84 ejaculates of 5.349 x 10^9 sperm. The sperm concentration was estimated to be less than 100 x10^6 sperm/mL for 67 of 84 ejaculates collected.

Estimated testicular volume for the stallion was 225 ml. If testes were producing sperm at a normal rate, daily sperm output was expected to be 4.6 x 10^9 sperm. Four ejaculates were collected once daily from the stallion to stabilize extragonadal sperm reserves. Total sperm number in the fourth ejaculate was 3.67 x 10^9 sperm, suggesting spermatogenic efficiency ($\leq$ 80%) was below normal. Among all four ejaculates, the percentage of progressively motile sperm averaged 27% and the percentage of morphologically normal sperm averaged 38%. The most common morphologic defects were abnormally shaped midpieces (30%) and bent tails (19%). Semen from three ejaculates was processed by cushioned centrifugation or cushioned centrifugation followed by density gradient centrifugation in EquiPure™ (Bottom Layer). Semen was evaluated for sperm motion characteristics immediately after each processing step and after 24 hours of cooled storage. The effect of seminal plasma was also evaluated. The sperm morphologic profiles of unprocessed (raw) semen and EquiPure™-processed semen were also compared. The percentage of morphologically normal sperm increased from 40% to 76% after density-gradient centrifugation, primarily due to reduced percentages of abnormal (irregular or bent) midpieces and bent tails. For this ejaculate and others evaluated from this stallion, sperm velocity was increased (approximately doubled) when seminal plasma was replaced with that obtained from a known fertile

stallion. Sperm motility parameters in semen extended after density-gradient centrifugation and replacement of seminal plasma were maintained through 24 hours of cooled storage.

A fertility trial was conducted with semen from this stallion to determine if insemination of mares with EquiPure™-processed semen would yield commercially acceptable pregnancy rates. Ten reproductively normal mares were inseminated as follows: 1) five mares were inseminated once with 100 x 10^6 total sperm, and 2) five mares were inseminated once with 200 x 10^6 total sperm. The seminal plasma was replaced with that of a known fertile stallion. Seminal plasma from the donor stallion was procured by centrifuging raw semen at 1000 x *g* for 15 min, followed by filtration of supernate through tandem 5.0- and 1.2-µm pore-size nylon filters to remove any remaining sperm from the seminal plasma. One-mL aliquots of seminal plasma were frozen in vials at -80°C until used. Inseminate volumes ranged from 0.25 to 0.58 mL. A transrectally-guided deep horn insemination technique was used. The pregnancy rate per cycle was 100% (5/5) for mares inseminated with 100 x 10^6 processed sperm and 80% (4/5) for mares inseminated with 200 x 10^6 processed sperm. Two mares, one in each treatment group, experienced double ovulations, and each of these mares was diagnosed with twin pregnancies. As such, pregnancy rate per ovulation was 100% (6/6) for mares inseminated with 100 x 10^6 processed sperm and 83% (5/6) for mares inseminated with 200 x 10^6 processed sperm. Based on the post-density gradient centrifugation recovery rate of sperm in this trial, the stallion would have had produced sufficient processed sperm to breed 17 mares if mares were inseminated with 100 x 10^6 processed sperm.

For the following breeding season, 212 mares were inseminated - primarily using fresh EquiPure™-processed semen with seminal plasma replaced with that from a fertile donor stallion. Chilled transported semen was not offered during this season because there were in excess of 60 re-breed contracts expected from the previous season, and demand for breedings on any given day early in the season would be high. Pregnancy rates per cycle and per season were 62% and 91%, respectively. Mares were inseminated with 0.5-2.0 mL doses of EquiPure™-treated semen with a transrectally guided deep-horn technique. The lowest dose contained 98 x 10^6 sperm. The maximum number of mares bred with one ejaculate was 11.

This stallion has become a successful sire and has produced as many as 220 pregnancies in subsequent years. Chilled transported semen has been added to the program and he currently breeds more mares away from the farm than on-site. EquiPure™-processed semen and cushion centrifuged and re-extended semen are currently utilized to cover shipment orders on a day-to-day basis.

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Effects of a depot progestin on spermatogenesis in postnatal pigs

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The search of alternatives for sterilization of boars has been the most recent trend for research for animal welfare activists. Although surgical castration has been the normal practice on swine farms to eliminate the possibility of boar taint, concerns are being raised regarding the pain associated with the surgical removal of testes. The objective of the study was to investigate chemical sterilization of pigs as an alternative to surgical castration. The primary goal was to determine if a single exposure of a subcutaneous administration of a depot progestin, depot medroxyprogesterone acetate (Depo Provera®, Pfizer Inc., New York, NY) in postnatal pigs will eliminate Leydig cell proliferation and production of high testosterone levels that can result in boar taint. Additionally, seminiferous tubules were evaluated at the end of the study to determine the effects of the depot progestin on spermatogenesis in the peri-pubertal boars. Crossbred boars (n=15) were administered a single injection of time-released progestin at 10 weeks of age and monitored weekly and biweekly for serum testosterone and androstenedione levels as well as testis size measured by calipers. Control boars were treated with a saline injection at 10 weeks of age. Animals were sacrificed at 26 weeks of age and testicular tissue obtained and fixed in Bouin's fixative, embedded in paraffin, sectioned, placed on slides and stained with hematoxylin and eosin. Data from weekly and biweekly collections were analyzed using a repeated measures MANOVA using SAS (SAS 9.1, SAS Inc., Cary, NC). A chi-square analysis was performed to compare number of spermatids found in the lumen of the seminiferous tubules. The results of this project reveal that testis size of postnatal boars treated with medroxyprogesterone acetate is significantly decreased ($P < 0.001$) over time compared to control boars. The reduction in testis size was not pronounced enough that personnel could not tell that it was an intact male. Testosterone and androstenedione assays via radioimmunoassay indicated that both hormone levels were decreased significantly ($P < 0.05$) for 4-6 weeks post-treatment, but then returned to pre-treatment levels and were comparable to control boars. Additionally, body weights of the treated and control boars remained similar ($P > 0.05$) throughout the study. Histological evaluation of the seminiferous tubules revealed a trend ($P = 0.06$) for diminished spermatogenesis with the treated boars having fewer spermatids. These results confirm that synthetic progesterone can suppress testosterone production, testis development of treated animals, but these effects are reversible as the boar reaches puberty and the weight for slaughter in the U.S. Correct timing and dosage for treatment of postnatal pigs with depot progestin still needs to be evaluated.

Keywords: Swine, depot, progestin, castration, medroxyprogesterone acetate.

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Effect of administration of oxytocin during diestrus on the duration of corpus luteum function and estrous behavior in cycling mares

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It has been demonstrated that intramuscular (IM) administration of 60 units of oxytocin once[1] or twice[2] daily on days 7 to 14 after ovulation induced prolonged corpus luteum (CL) function in approximately two thirds of mares treated, making it a plausible method of suppressing estrus. The objective of this study was to monitor CL function and estrous behavior in mares for 90 days after administration of oxytocin during diestrus.

Jugular blood samples were collected every other day on days 0 (ovulation) to 30 and then three times weekly (M, W, F) until day 90 for determination of serum progesterone concentration. On day 7, mares were randomly assigned to saline-treated control and oxytocin-treated groups (n = 9/group with eight light horse breed and one heavy horse per group). Beginning on day 7, control mares received 3 cc sterile saline IM and oxytocin-treated mares received 60 units (3 cc) oxytocin IM once daily through day 14. Mares were exposed to a stallion for estrus detection on the same schedule as blood sample collection. Fifteen specific behavioral responses were recorded, six reflecting non-receptivity and nine reflecting receptivity, as the basis of judgment of *ambivalent, mild diestrus, strong diestrus, weak estrus,* or *good estrus*. Mares were considered to have prolonged CL function if progesterone remained >1.0 ng/ml continuously through day 30.

Two of nine control (22%) and six of nine oxytocin-treated (67%) mares had prolonged CL function (P=0.08). The mean (± sem) duration of CL function in the two control mares with prolonged CL function was 77.5 ± 0.2 days and in the six oxytocin-treated mares was 68.8 ± 4.1 days. In both of the control mares and one of the six oxytocin-treated mares with prolonged luteal function, estrus was not observed while progesterone remained above 1.0 ng/ml. For the remaining five oxytocin-treated mares with prolonged luteal function, weak estrus was observed during the period of elevated progesterone (11 to 65 days before progesterone was last measured above 1.0 ng/ml), which corresponded with a range of 13 to 59 days after ovulation (mean of 44.8 ± 8.4 days). In summary, oxytocin treatment effectively prolonged CL function for approximately two months in two-thirds of the treated mares, and somewhat surprisingly during the period of prolonged CL function weak estrous behavior was observed.

Keywords: Equine, mare, oxytocin, corpus luteum, estrous behavior

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Effects of thawing time on membrane integrity and motility of frozen-thawed canine spermatozoa using commercial semen extenders

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Hypothesis

Our hypothesis was that a commercially available canine extender, compared with a human extender, using our current cryopreservation protocol would yield increased post-thaw motility and intact membranes when thawed at 50° for 10 sec or at 37° for 30 sec. The specific aims were to determine how two different thawing times and temperatures affected post-thaw motility of canine semen cryopreserved with two different commercial extenders.

Experimental methods

A single ejaculate was collected from 11 mature dogs of different breeds. Each ejaculate was prepared for cryopreservation with two commercial extenders, Irvine (Irvine Scientific, Santa Ana, CA; IRV) or Partnar (Port Huron, MI; PAR). After a 10 min centrifugation at 900 x g and aspirating the supernatant, each extender was added and each sample was cooled 60 min before adding more extender containing 12% glycerol (yielding a final glycerol concentration of 6% and 50 x 10^6 cells/ml). Each of the four aliquots was immediately loaded into 0.5 mL straws, placed on a boat 4 cm over liquid nitrogen for 10 min, and then plunged and maintained at -196° C for at least seven days before thawing. For each aliquot, two different thaw protocols were used; 50°C for 10 sec (50) or 37°C for 30 sec (37). Five minutes after thawing, each sample was assessed for total (TM) and progressive (PM) motility using a computer assisted sperm analyzer (SpermVision, Minitube, Verona, WI) and for membrane integrity (IM) using SYBR-14/PI (LIVE/DEAD® Sperm Viability Kit, Invitrogen™, Carlsbad, CA). A repeated measures analysis in an ANOVA of a 2^3 factorial arrangement of treatments with dog as a random effect in a mixed effects model using the SAS mixed procedure was performed. Pair-wise t-tests of least squares means were performed as follows, (IRV37, IRV50), (PAR37, PAR50), (IRV37, PAR37), and (IRV50, PAR50).

Results

For TM, the values (mean±SE) were 51.5±4.4, 55.9±3.4; 36.9±3.9; and 43.5±4.1 for the IRV37, IRV50, PAR37 and PAR50, respectively. For PM the values (mean±SE) were 45.2±5.0, 49.6±4.1, 31.8±4.5 and 36.7±4.6 for the IRV37, IRV50, PAR37 and PAR50, respectively. For IM the values (mean±SE 4.0) were 56.3±3.7, 51.8±3.2, 40.6±2.8 and 44.0±3.3 for the IRV37, IRV50, PAR37 and PAR50, respectively. The IRV50 had significantly greater TM ($P = 0.0072$) and PM ($P = 0.0037$) than the PAR50, but not the IRV37 (TM $P = 0.330$, PM $P = 0.3112$). The PAR50 did not differ significantly in TM ($P = 0.1452$) or PM ($P = 0.2546$) than the PAR37. The IRV37 had significantly greater TM ($P = 0.0017$) and PM ($P = 0.0026$) than the PAR37. The IM were greater for the IRV37 than the PAR37 ($P=0.0611$), but there were no significant differences among the other IM comparisons.

Conclusions

There were no differences within extenders for TM, PM or IM, however the IRV had significantly greater values than the PAR for all parameters. Our hypothesis was rejected and changing the extender or thaw protocol offered no advantage over the current protocol.

Keywords: Canine; semen; cryopreservation; sperm; motility; membrane integrity

Effects of estradiol on uterine blood perfusion in reproductively healthy mares and mares affected with uterine vascular elastosis

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Uterine vascular elastosis is a degeneration of the uterine vasculature that is associated with aged, multiparous, infertile or subfertile mares. In previous experiments, we associated this degeneration with reduced uterine blood perfusion (UBP). Histologically, these lesions consist of enlargement, duplication and thickening of the membrana elastica interna of the uterine blood vessels. We hypothesize that vasodilatation is impaired in affected uterine vasculature. To test the functionality of these vessels we evaluated the effects of 17□estradiol (E_2) on UBP in reproductively healthy mares and mares affected by uterine vascular elastosis.

Mares with normal uterine vasculature or mild changes were used as controls and further divided by stage of cycle (determined by ultrasound examination and confirmed by circulating serum progesterone levels; <0.5 ng/mL for estrus and >1.0 for diestrus) into control-estrus (n=3) and control-diestrus (n=3). Mares affected with severe uterine vascular degeneration were used for elastosis groups, also divided into elastosis-estrus (n=3) and elastosis-diestrus (n=3). Fluorescent microspheres (FM; 15um diameter, Triton Technology, Inc, San Diego CA) were injected directly into the left ventricle of the heart of anesthetized mares and were used to determine baseline levels of UBP using a reference blood sample method using four different arterial blood samples to ensure adequate mixing and sampling, followed by the administration of 1.0 mg/kg of E_2 (IV). After an onset period of 90 minutes, FM with a different fluorescent dye were also injected and used to determine changes in UBP induced by E_2. Mares were euthanized and reproductive tract removed. Concentrations of the two different FM (with different excitation and emission wavelengths) were determined, by digesting the tissue, recovering the FM, reading the fluorescent intensity (proportional to the number of FM and used to calculate UBP levels. Repeated measures were used for comparison of baseline levels and post-estradiol levels and a 2x2 ANOVA was used for comparison between vascular grade and stage of cycle.

Results are expressed as baseline levels of UBP and post-E_2 UBP levels (mL of blood/min/100 g of uterine tissue ± SD). Control mares during estrus had an overall increase in UBP ($p < 0.05$). Baseline UBP levels were 18.8 ± 2.9 mL/min/100 g vs. post-E_2 UBP levels 28.3 ± 2.9 mL/min/100g. No other group had an overall significant increase when comparing baseline UBP levels vs. post- E_2 UBP levels (control-diestrus: 12.1 ± 2.9 vs. 14.0 ± 3.1, elastosis-estrus: 6.8 ± 1.2 vs. 6.7 ± 2.6, elastosis-diestrus: 7.2 ± 2.4 vs. 7.4 ± 2.4). The baseline UBP of the control-estrus group was also significantly different of all other groups. Additionally, data were analyzed by uterine region (uterine horns, uterine body and cervix) and expressed as per cent of change of UBP from baseline levels ((post- E_2 UBP – baseline UBP / baseline UBP) x 100 ± SD. In the control-estrus group, there was an increase in UBP in both uterine horns 52.4 ± 36% ($p=0.065$), 54.4 ± 20% ($p < 0.05$) and uterine body 54.2 ± 17 % ($p < 0.05$). In the control-diestrus group, there was an increase in both uterine horns; 25.4 ± 5% ($p < 0.05$) and 32.9±5 % ($p < 0.05$). No uterine region had a significantly increased of UBP after E_2 administration in mares affected by uterine vascular elastosis.

The difference in vasodilatory response induced by estradiol between reproductively healthy mares and mares affected with elastosis of the uterine vascular bed indicates that the vasodilation of the affected vessels is compromised.

Keywords: Uterine blood perfusion, elastosis, infertility, mare, estradiol.

Association of IGF-1 concentrations in seminal plasma and puberty in Gir Zebu bulls

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The objectives of this study were to determine whether IGF-1: i) was expressed in the seminal plasma of peripubertal bulls, ii) could be used as a seminal biomarker for puberty, iii) seminal plasma concentrations could be correlated with breeding soundness examination (BSE) scores and seminal plasma proteins. Semen was obtained monthly from dairy Gir zebu bulls (n=16; 14 to 26 mo old) by electroejaculation. At each collection, all animals were weighed and underwent a complete BSE including: scrotal circumference (SC), sperm motility (MOT), sperm concentration (CONC), seminal volume and sperm morphology (MORPH). The bulls were ranked using a BSE scoring system for zebu breeds (Vale Filho, 1986), where MORPH and SC are scored 0 to 40 pts and MOT 0 to 20 pts. Seminal plasma was harvested (centrifuged 600xg/10min), extended 1:1 (v/v) into a buffer (Tris, CaCl2, NaN2, Pepstatin-A, PMSP,), and preserved in LN_2. Puberty was defined as described by Wolf et al. (1965). The data were adjusted for age at puberty according to Brito et al. (2004). Seminal IGF-1 was analyzed by RIA and seminal plasma proteins were characterized using SDS-PAGE gels. Data analyses were carried out using SAS (2002). Under these data adjustments, all variables were compared between periods. After adjusting the data according to age in relation to puberty (days) at time 0 (zero) it was carried out a frequency distribution of the ages of the 16 animals using the FREQ procedure. It was adopted as a point of separation of the groups the median, 18 months of age at the time. Thus, animals with age at puberty below the median were considered as precocious (n=8) and those above the median considered regular (n=8). The concentration of IGF-1 was tested for normality using UNIVARIATE procedure. Correlations between parametric variables were estimated by Pearson's correlation coefficient and the associations between the parametric and the nonparametric variables by the Spearman correlation coefficient. Results showing no statistical difference between groups and between sampling periods were pooled for joint analysis. The significance was set at $p<0.05$. Using SDS-Page gels, 37 bands (6.9 to 236 kDa) were identified, whereas the presence of the bands 112, 27, 18, 12, 11 and 6.9 kDa was significant ($p<0.05$) and positively correlated with earlier puberty onset (r=0.57 to 0.62), BSE scores (r=0.55 to 0.59) and with IGF-1 concentrations (r= 0.56 to 0.6), whereas the bands 55, 47 and 25kDa presented negative correlations (r= - 0.4 to - 0.6) with puberty onset and IGF-1 and BSE scores. There was positive correlation (r= 0.65) between seminal IGF-1 concentration and BSE score. There were positive correlations (r = 0.7 to 0.82) between body weight and BSE score. In summary, seminal IGF-1 was expressed in increasing concentrations from -60 to puberty onset. Additionally, once IGF-1 presented positive correlation with BSE scores and seminal plasma proteins, we suggest that IGF-1 could be used as indicator of semen quality and puberty onset for zebu dairy bulls.

Keywords: Zebu, breeding soundness examination, puberty, seminal plasma.

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Removal of seminal plasma by filtration prior to cryopreservation of canine sperm

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Some cryopreservation protocols for dog sperm include removal of seminal plasma by centrifugation. However, centrifugation can potentially induce damage to the sperm of some dogs and requires costly equipment. A sperm filter has been recently developed to remove seminal plasma from stallion semen[1] and its use yielded comparable results to centrifugation. Therefore, our hypothesis was that removal of seminal plasma by filtration would improve canine sperm cryosurvival when compared to centrifugation. Objectives of the study were to determine whether or not removal of seminal plasma by filtration or centrifugation would: 1) yield similar sperm recovery, motility and viability prior to cryopreservation; and 2) affect sperm motility and viability post-thaw. Two ejaculates from each of four dogs were collected, diluted 1:1 (v:v) with freezing extender (CanFreeze - Step 1®; Partnar Animal Health, Port Huron, MI), and divided into two aliquots. Aliquots were then centrifuged (900 x g for 8 min) or filtered through a sperm filter (Botupharma Ltda., Botucatu, Brazil). Sperm were then resuspended in the same extender and frozen according to the manufacturer's instructions. Sperm concentration, motility and viability were evaluated before and after seminal plasma removal, and after thawing. Total (TM) and progressive (PM) motility were evaluated by Computer-Assisted Sperm Analysis (CASA; Ceros®, Hamilton Thorn Biosciences, Beverly, MA). Sperm concentration and plasma membrane integrity (i.e. viability) were determined using an automated cell counter (NucleoCounter®, ChemoMetec A/S, Denmark) and staining cells with propidium iodide. Data were analyzed by ANOVA for repeated measures and significance was set at $P<0.05$. Data are expressed as mean percentage ± SEM. There were no differences in sperm recovery (95±4 *vs.* 91±5), TM (89±2 *vs.* 89±1) and viability (86±1 *vs.* 87±1) between centrifugation and filtration methods, respectively. Progressive motility was significantly decreased after removal of seminal plasma by both centrifugation (63±2) and filtration (63±2), compared to pre-processing motility (75±3). Cryopreservation significantly decreased post-thaw TM, PM, and viability, regardless if samples were submitted to centrifugation (48±5, 33±4 and 53±7) or filtration (41±3, 27±2 and 53±6), respectively. Lastly, post-thaw TM and PM were similar between centrifugation (35±9 and 22±7) and filtration (30±6 and 18±5) after incubation at 37 °C for 30 min.. In summary, removal of seminal plasma by filtration was as effective as centrifugation and resulted in similar sperm survival variables post-thaw. Filtration is a potential alternative to centrifugation when equipment is limited. Future studies will focus on the use of filtration for processing semen from dogs whose sperm are more sensitive to cryopreservation.

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Comparison of vaginal flora in estrous dogs with those of spayed dogs and dogs with recurrent urinary tract infections

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Infectious urogenital diseases represent a major source of morbidity in both intact and spayed bitches. Pyometra represents one of the major diseases of intact bitches, while urinary tract infections are a major source of disease in spayed bitches. The vaginal flora is a major component of the defense mechanism of the lower urogential tract of the female, protecting against both urinary and uterine infections.[1] However, no study has previously attempted to broadly characterize the vaginal flora in different groups of dogs. The current study aimed to characterize the vaginal flora of intact estrous bitches, healthy spayed bitches and spayed bitches with recurrent urinary tract infections (rUTIs). Based on previous studies we hypothesized that estrous bitches would have the most diverse bacterial flora, while dogs with rUTIs would have the highest incidence of *Escherichia coli* and the lowest incidence of lactic acid producing bacteria (LAB).

Samples were collected using a sterile, double-guarded swab from the cranial vagina of healthy estrous bitches (Group E; n=11), healthy spayed bitches (Group Sp; n=23) and spayed bitches with recurrent urinary tract infections (Group rUTI; n=11). Aerobic bacteria were cultured using standard procedures. Selective subculture for LAB was performed using Rogosa agar under microaerophilic and anaerobic conditions. Identification of *Lactobacillus* species via 16sS rRNA gene sequencing will be completed at the termination of the study. *Mycoplasma* ssp. were cultured in Frey's broth with 15% swine serum, and identification was confirmed by 16S rRNA gene PCR. Numbers of bacterial genera were compared between groups using a one-way ANOVA. Prevalence of specific organism was compared between groups using a 2x3 Fisher Exact Test.

In total, 75 organisms were cultured in 45 dogs. The average number of cultivated bacterial genera was 2.1 for dogs in Group E, 1.6 for dogs in Group Sp and1.9 for dogs in Group rUTI (P=0.2). The prevalence of *E. coli* was lowest for Group Sp, but no statistical differences were detected (45, 13, 27% respectively for Groups E, Sp and rUTI; P=0.13). The prevalence of LAB was not different between groups (9, 22, 0% respectively for Groups E, Sp and rUTI; P=0.23). The prevalence of *Mycoplasma* ssp. was higher in estrus dogs than in either healthy spayed or rUTI dogs (54, 13, 0% respectively for Groups E, Sp and rUTI; P=0.005). In conclusion, differences in flora were found between intact estrous and spayed bitches. It is not known whether these differences were the result of the bitch's stage of cycle (estrus or anestrus) or reproductive status (intact or spayed). Work is underway to further characterize these differences and determine the effect of oral probiotics on canine vaginal flora.

Keywords: Canine, vaginal flora, Mycoplasma, Lactobacillus

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Three-day progesterone priming protocol for breeding goats during anestrus/transition

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Many traditional protocols used to breed goats during anestrus and transition call for extended periods of progesterone priming lasting anywhere from nine to 21. Work in our laboratory has shown that four-cell embryos can be reliably produced outside of the breeding season using a three-day progesterone priming protocol. We hypothesized that this protocol could be modified to induce estrus for breeding goats during anestrus and transition. The aim of this study was to determine the efficacy of this short progesterone priming protocol to induce estrus and produce viable pregnancies. Progesterone was administered using controlled internal drug release (Eazi-Breed CIDR Sheep & Goat; Pharmacia and Upjohn, New York, NY) inserted into the vagina for three days. Follicle stimulating hormone (Follitropin-V; Bioniche, Belleville, Ontario, Canada) was administered on the second and third days of progesterone priming (32 mg IM, SID). Prostaglandin F2α (Lutalyse; Pfizer, New York, NY) was given (5 mg IM) on the day of CIDR removal to eliminate any corpora lutea (CLs). Gonadotropin releasing hormone (GnRH; Cystorellin; Merial, Duluth, GA) was given (50 mcg IM) two days after CIDR removal to induce ovulation. All does were naturally bred by the same buck at the first sign of estrus and every twelve hours thereafter until they were no longer receptive. Control animals received the same treatments as the experimental does, except for the CIDR. Blood was drawn daily during the priming protocol and twice weekly during the first four weeks of gestation to determine serum progesterone (P4) levels. Although the short priming protocol induced behavioral estrus with successful breeding in the majority of the treatment animals, only one animal carried a pregnancy to term. The data are shown below.

Protocol	Estrus (%)	Breeding (%)	Pregnant (%) 37 days	Pregnancy to term (%)
Treatment (n=10)	9/10 (90%)	7/8* (87.5%)	3/7 (42.9%)	1/3 (33.3%)
Control (n=3)	2/3 (66.7%)	2/2 (100%)	0	0

*One animal was not bred due to small size

Serum P4 levels for the pregnant animals were 11.9-24.7 ng/mL 9 days post-breeding compared to 5ng/mL or less for non-pregnant animals. By 12 days post-breeding P4 levels were dropping in all pregnant animals, to 3.2-4.9 ng/mL at 31 days, suggesting possible problems with CL function. The results of this study suggest that although the three-day progesterone priming protocol was successful in inducing estrus adequate for breeding, the resulting pregnancy rates were low. In light of the small numbers used in this study, further research involving more animals is needed to investigate if the problems seen in progesterone levels during early pregnancy are specifically related to the short progesterone priming protocol, or are in general, inherent to breeding goats during anestrus and transition.

Keywords: Goat, CIDR, estrus synchronization, progesterone, anestrus

Effects of lactoferrin on stallion sperm survival and function in vitro

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Lactoferrin is an iron-binding glycoprotein found in many biological secretions including blood, tears, milk and saliva. Our laboratory demonstrated that lactoferrin reduces post-breeding uterine inflammation in mares by decreasing expression of pro-inflammatory cytokines. Therefore, lactoferrin could potentially be incorporated into commercially available semen extenders to modulate the uterine inflammation post-breeding. Our hypothesis was that addition of lactoferrin to semen extender would not be detrimental to sperm survival and function. Our objective was to determine the effects of lactoferrin on stallion sperm motility and viability during 48 h of storage at 5° C. Four ejaculates from each of four stallions, on a regular collection schedule, were collected, diluted 1:1 (v:v) with skim milk-based extender without antibiotics (EZ-Mixin® BF, Animal Reproduction Systems, Chino, CA), and centrifuged at 400 x g for 12 min to remove seminal plasma. Sperm were then resuspended in the same extender containing 0 (control), 10, 20, and 30 mg/mL of lactoferrin and stored at 5° C for 48 h. Total (TM) and progressive (PM) motility were evaluated subjectively and by Computer-Assisted Semen Analysis (CASA; Ceros®, Hamilton Thorn Biosciences, Beverly, MA) at 0, 24 and 48 h of storage. Plasma membrane integrity (i.e. viability) and growth of microorganisms were evaluated at 24 h of incubation. Sperm viability was determined by exclusion of propidium iodide stain using an automated cell counter (NucleoCounter®, ChemoMetec A/S, Denmark). Individual microorganism growth was scored from 0 to 5 (0 = no growth; 5 = heavy growth) according to the number of colonies observed after 48 h of aerobic incubation. When multiple organisms were isolated from a sample, the sum of the growth scores for each microorganism was used as the sample's score. Data were analyzed by ANOVA for repeated measures and significance was set at $P<0.05$. Data expressed as mean ± SD. TM and PM were similar between control and all lactoferrin groups at times 0 and 24 h of storage. However, a significant decrease in TM and PM was observed at 48 h of storage when lactoferrin was present at 20 mg/ml (TM = 47±17%; PM = 24±14%) and 30 mg/ml (TM = 44±17%; PM =22±12%), compared to control (TM = 61±20%; PM = 38±18%). The percentage of sperm with intact plasma membrane after 24 h of storage was similar between all groups. Growth of microorganisms was significantly reduced when lactoferrin was present at 20 mg/ml (5±3) and 30 mg/ml (4±3) compared to control (12±5). In conclusion, lactoferrin did not affect sperm motility and viability during the first 24 h of storage and was effective in reducing the growth of microorganisms. Potentially, addition of lactoferrin to semen extenders at concentrations <20 mg/ml may allow modulation of uterine inflammation post-breeding, particularly in mares susceptible to mating-induced endometritis. Future studies in our laboratory will focus on the effects of lactoferrin on fertility of mares susceptible to endometritis.

Keywords: Lactoferrin, stallion, semen, spermatozoa

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Authors are thankful to Animal Reproduction Systems for providing supplies and DMV International for providing lactoferrin.

Keywords: Lactoferrin, stallion, semen, spermatozoa

Sperm plasma membrane integrity during sperm extra-gonadal reserve depletion in stallions

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The sperm plasma membrane integrity during equine sperm extra-gonadal reserve (EGR) depletion has not been previously investigated. Knowing when stored sperm obtain their greatest plasma membrane quality after semen has been collected daily may improve sperm utilization. The objectives of the study were to estimate sperm output and evaluate the equine plasma membrane during sperm EGR depletion in healthy stallions. We hypothesized that the sperm plasma membrane integrity will improve during sperm EGR depletion.

Six light breed sexually rested stallions were collected daily for seven days to deplete the EGR. On collection days 1, 3, 5 and 7, a semen sample was obtained for sperm concentration and evaluation of plasma membrane integrity. A hemacytometer was used to estimate sperm concentration and the fluorescent probes SYBR-14/PI (Sperm Viability Kit®, Molecular Probes Inc., Eugene, OR) were used to evaluate the sperm plasma membrane integrity. A total of 10,000 cells, in triplicates, were analyzed for fluorescence using a flow cytometer (Accuri C6, BD Accuri Cytometers, Ann Arbor, MI). The data were examined for normality (Shapiro Wilk's; $p < 0.05$) and analyzed for the effect of day on sperm output and plasma membrane integrity. The daily sperm output data were fitted into a simple linear regression equation. The plasma membrane data were analyzed using a one-way ANOVA (SAS 9.3) and where a significant effect ($p < 0.05$) of day was observed, Tukey's test for multiple comparisons was applied.

The sperm concentration and plasma membrane data followed a normal distribution. The regression equation predicting sperm output (X) for stallions collected daily during sperm EGR depletion was: $\log (X) = 8.95 \times 10^9 - 0.185 \times 10^9 * (\text{day})$. Intact plasma membrane percentage (mean ± SE) were 61.6 ± 3.34^a, 70.7 ± 3.23^b, 71.4 ± 2.28^b and 69.1 ± 2.57^b for day 1, day 3, day 5 and day 7, respectively. There was a significant difference in intact sperm plasma membrane between day 1 and days 3, 5 and 7, but no significant difference between days 3, 5 and 7.

We can conclude that the daily sperm output of sexually rested stallions during the sperm EGR depletion period may be predicted using the above regression equation. The plasma membrane integrity of equine sperm improved from the first collection (day 1) to the third collection (day 3); however, thereafter no improvement of sperm plasma membrane was observed. This indicates that during sperm EGR depletion, the percentage of sperm with intact plasma membrane have reached their greatest at day 3 and have the same plasma membrane quality as sperm collected after the sperm EGR depletion.

Keywords: Sperm, plasma membrane, stallion, extra-gonadal sperm reserve, sperm output

Induction of hyperactivation in stallion sperm using 4-amynopyridine

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Hyperactivation is a change in the pattern of sperm motility required for fertility. Procaine-treated stallion sperm evaluated by computerized-assisted sperm motility analysis (CASA) became hyperactivated as defined by decreases in straight line velocity (VSL) and linearity (LIN), and increases in amplitude of lateral head displacement (ALH) and curvilinear velocity (VCL). This also yielded high rates (~60%) of homologous in vitro fertilization (IVF). Because procaine may be toxic to oocytes, alternative hyperactivation-inducing treatments are desirable. Thus, our hypothesis was that 4-aminopyridine (4-AP), which raised calcium levels in human and mouse sperm and hyperactivated mouse sperm, would induce stallion sperm hyperactivation. Sperm (3 stallions x 2 ejaculates) were diluted in modified Whitten's (37°C) and treated with: 5 mM procaine; 2, 4, 6, 8 or 10 mM 4-AP; or, untreated. Sperm motility measures obtained via CASA were analyzed by ANOVA with Fisher LSD test applied for mean separation ($P<0.05$). Notably, 4 mM 4-AP yielded motility changes similar to procaine ($P>0.05$) and consistent with hyperactivation as compared to untreated controls ($P<0.05$): VSL = 52.3±23.9 vs. 87.9±12.1 μm/sec; LIN = 21.6±5.1 vs. 52.4±8.4 %; ALH 9.1±2.8 vs. 6.3±0.8 μm/sec; and, VCL = 256.7±74.1 vs. 192.6±31.3 μm/sec, respectively. We conclude that inducing hyperactivation with 4-AP may be an alternative to procaine for supporting equine IVF.

Keywords: Stallion sperm, hyperactivation, 4-amynopyridine, procaine, CASA.

Effect of electroejaculation on behavioral and hormonal indicators of stress and nociception in beef bulls

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Electroejaculation has attracted attention as an animal well-being issue. Substance P is an eleven-peptide neurokinin involved in the integration of stress, pain, and anxiety. Plasma concentrations of substance P were elevated and associated with vocalization following castration compared to sham-castrated calves while cortisol concentrations were not different,[1] suggesting substance P may be more specific for assessment of pain. We hypothesized that substance P would be a more specific indicator of nociceptive stress following electroejaculation as compared to cortisol, progesterone, and vocalization. The objective of this study was to determine changes in hormonal and behavioral indicators of stress and nociception following electroejaculation in bulls. Nine Angus bulls (15 ± 0.76 months, 501.9 ± 14.3 kg) received each of three treatments in three 3x3 Latin squares. Treatments (applied at time 0) included no manipulation (control), rectal probe insertion without stimulation (probed), and electroejaculation (EEJ, Lane Pulsator IV™, one complete automated cycle); three bulls were treated contemporaneously (one in each group). Blood was collected via indwelling jugular catheter at -60, -30, 0, 2, 10, 20, 30, 45, 60, 75, 90, and 120 minutes relative to treatment. Vocalization (yes/no) was recorded. Concentration of plasma cortisol, progesterone, and substance P immunoreactivity were determined by competitive enzyme-linked chemiluminescence, radioimmunoassay, and ELISA, respectively. Concentrations of plasma hormones were analyzed by a mixed model analysis of variance for repeated measures; vocalization was analyzed by Fisher's Exact Test. A greater number of bulls ($P=0.029$) vocalized during electroejaculation (5 of 9; 55.6%) compared to controls (0 of 9; 0%). There was an effect of treatment and an interaction of treatment and time on concentrations of plasma cortisol (treatment, $P=0.0013$; treatment*time, $P<0.001$) and progesterone (treatment, $P=0.0012$; treatment*time, $P<0.001$), with higher concentrations in EEJ compared to probed and control groups; elevations persisted through the 45 minute sample. Mean plasma concentration of substance P was not different ($P=0.6264$) between the three treatment groups. Increased vocalization and plasma concentrations of cortisol and progesterone indicate an acute stress response following electroejaculation; however, there is no difference in plasma concentration of substance P, suggesting the stress is not due to nociception.

Keywords: electroejaculation, bull, substance P, nociception, stress

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Effects of decreasing doses of follicle-stimulating hormone on multiple ovulations and embryo production in alpacas

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Alpacas have a gestation length of nearly one year, and therefore females can only produce one offspring per year. In order to accelerate the genetic gain of a herd, superovulation and embryo transfer techniques can be used to produce multiple embryos from genetically valuable females where allowed by breed registries. Our hypothesis was that administration of decreasing doses of follicle-stimulating hormone (FSH) would induce growth of multiple preovulatory follicles resulting in multiple ovulations and increased embryo production. Our specific aim was to determine the effects of FSH administration in decreasing doses on follicular growth, ovulation and embryo production in alpacas. Females were teased with a male daily and transrectal ultrasonography was performed to determine follicular growth. When receptivity to a male was observed, females were naturally mated once to one of two fertile males and ovulation was confirmed by daily ultrasonography and/or teasing (Control Group; n = 15). Females in the treatment group (n = 13) received human chorionic gonadotropin (hCG; 1000 IU, IV) when a preovulatory follicle >7 mm was present to induce ovulation and emergence of a new follicular wave. Starting at 60 h post-hCG, females received twice daily intramuscular injections of FSH at decreasing doses (i.e. Day 1 = 50mg; Day 2 = 40 mg; Day 3 = 30; Days 4 to 7 = 20 mg). Administration of FSH was discontinued when half of the follicles in the growing cohort were ≥ 7 mm in diameter, or after seven days. On the last day of FSH administration, females received cloprostenol (187 μg, IM, twice) to induce luteolysis. Receptive females were bred twice (within 12 h) and received hCG (2000 IU, IV) at time of the first breeding. Embryo collections were performed 7 to 9 days after breeding by transcervical uterine lavage without manipulating the reproductive tract per rectum. Only cycles that resulted in breeding and ovulation were included in the data analysis (Control = 32 cycles; Treatment = 14 cycles). Data were evaluated by one-way ANOVA and Fisher's exact test. Significance was set at $P<0.05$ and data are presented as mean ± SD. In the treatment group, females received FSH for a total of 5.2±1 days. Females treated with FSH had a significantly higher number of ovulations per cycle (8.9±4.4) compared to spontaneously ovulating females in the control group (1.03±0.2). In addition, more embryos were collected per flush from females receiving FSH (2.9±3.1) compared to control (0.48±0.6). However, embryo collection rates per ovulation tended ($P=0.09$) to be higher for females in the control group (47%) versus FSH-treated (32%). In conclusion, administration of decreasing doses of FSH to alpacas was effective in increasing the number of ovulations and consequently, the number of embryos produced. Future studies will focus on improving the efficiency (i.e. embryos produced per ovulation) of superovulation protocols in alpacas.

Keywords: Alpaca, embryo transfer, superovulation, camelid, follicle-stimulating hormone

Expression of vascular endothelial growth factor A and its receptor Flt-1 in canine placenta

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Introduction

Vascular endothelial growth factor A (VEGFA) and its receptor Flt-1 have been studied extensively in primate placentas, where they have been proposed to play a role in trophoblast invasion.[1] In addition, placental Flt-1 mRNA expression has been shown to increase at term in humans.[2] No studies have investigated the expression of these factors in the canine placenta at the time of parturition. Dysregulation of these factors could, for example, contribute to abnormal post-partum subinvolution of placental sites. Therefore, the aim of this study was to examine VEGFA and Flt-1 mRNA expression in canine chorio-allantoic tissue at pre-term, pre-labor and during parturition. The hypothesis was that expression of VEGFA and Flt-1 will be greater in term (pre-labor or parturient) than in pre-term bitches.

Keywords: Canine, chorio-allantois, Flt-1, parturition, placenta, VEGFA

Methods

Following ovariohysterectomy at 61±1 days past the luteinizing hormone (LH) surge (pre-term; n=4), chorio-allantoic tissue was collected without the marginal hematoma and flash frozen in liquid nitrogen. Chorio-allantoic tissue was collected in the same manner following elective cesarean section at 64±1 days past the LH surge prior to first stage labor (pre-labor; n=3) and following natural delivery (parturient; n=3). Total RNA was isolated using the TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Quantitative RT-PCR was performed using primers and probes developed for canine VEGFA and Flt-1 (Applied Biosystems, Carlsbad, CA). Gene expression was normalized to 18S rRNA expression and the first parturient sample collected was used as a calibrator. Relative expression was calculated using the relative quantitation ($2^{-\Delta\Delta Ct}$) method. Statistical analysis was performed using a Student's *t* test in Excel (Microsoft, Redmond, WA). Significance was defined as $p<0.05$.

Results

Flt-1 expression was higher in tissues collected from pre-labor and parturient bitches than in tissues from pre-term bitches. However, there were no differences between groups in VEGFA expression.

Conclusion

Based on these results, VEGFA mRNA is constitutively expressed during late gestation and parturition in the dog. Its receptor, Flt-1 increases in the canine chorio-allantois at the time of parturition, similar to what has been reported in primates.[2] Flt-1 has been reported to be directly up-regulated by hypoxic conditions,[2] suggesting that placental hypoxia associated with parturition may lead to the observed up-regulation of Flt-1 during whelping.

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Effects of gonadatropin releasing hormone (GnRH) immunization in molly mules

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Introduction

Canine Gonadatropin Releasing Factor Immunotherapeutic® (canine GnRH vaccine, Pfizer Animal Health, New York, NY) is a vaccine labeled for the treatment of canine benign prostatic hyperplasia, which has been used to prevent estrous cyclicity and behavior in mares.[1-2] Serum assayed from these vaccinated mares had elevated GnRH antibody titers and progesterone concentrations consistent with anestrus (<1.0 ng/mL).[3] Female mules (mollies) display regular estrous cyclicity and behavior like the mare,[4] and therefore, the objective of this study was to determine whether GnRH vaccination would effectively prevent estrous cyclicity in mollies as well. We hypothesized that vaccinated mollies would develop GnRH antibodies, which would prevent estrous cyclicity and behavior.

Keywords: GnRH, mule, vaccination, progesterone, estrous behavior

Materials and methods

Six mature privately-owned anestrous mollies with a history of overt estrous behavior during the previous spring and summer were used for this study. All mollies were vaccinated with the canine GnRH vaccine (5 mL, IM) at 0 and 3 weeks in February and March. Jugular venous blood samples were collected at 0, 3, 7, 11, and 15 weeks. Sera were separated, aliquoted, and frozen at -20°C until analyzed. Gonadotropin releasing hormone antibody titers were determined using a previously validated ELISA with 1 μg/mL luteinizing hormone releasing hormone as the antigen (71447-49-9, Sigma). Serum progesterone levels were measured using enzyme-amplified chemiluminescence (Immulite® 1000, Diagnostic Products Corporation, Los Angeles, CA) and owners were asked to evaluate reproductive behaviors. Data were analyzed as a repeated measure in time design using PROC MIXED in SAS (V. 9.2, SAS Institute Inc., Cary, SC). Significance was defined as $p<0.05$.

Results

There were no adverse reactions to vaccination in any of the mollies. All developed GnRH antibody titers that were significantly higher at week 7 compared to week 0 and 15. Serum progesterone concentrations remained consistent with anestrus (<1.0 ng/mL) post-vaccination. Owners reported that non-vaccinated mares co-housed with mollies displayed estrous behavior, but vaccinated mollies did not display estrous behavior.

Conclusion

These results provide evidence that administration of the canine GnRH vaccine can be an effective way of preventing estrous cyclicity and behavior in mollies. Further research with a larger sample size and a double-blind study protocol is needed.

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Immunohistochemical analysis of gonadotrophin and kisspeptin fibers in the canine hypothalamus

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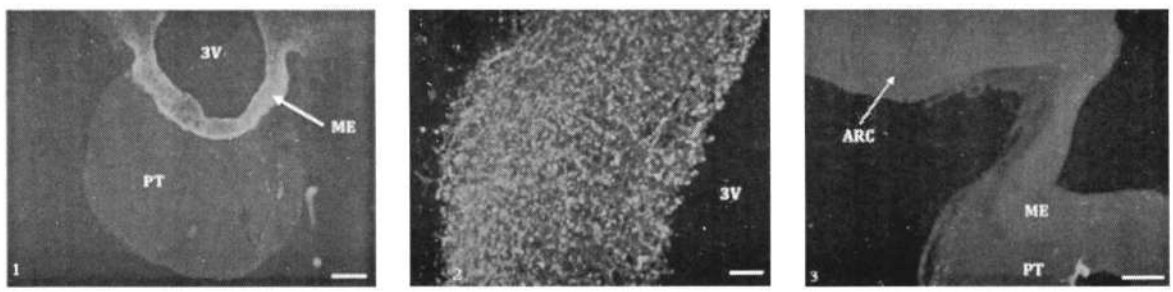
Evaluation of the interaction of gonadotrophin releasing hormone (GnRH) and kisspeptin (Kp) as peptides that modulate the higher order control mechanisms in reproductive physiology has been a recent development. A novel investigation into the anatomical location and possible interaction of GnRH and Kp neurons in the canine brain was undertaken with immunohistochemical (IHC) evaluation of domestic canids. The aim of this study was to characterize the location of GnRH and Kp neurons within the canine hypothalamus and to assess their interactions.

Four canines were humanely euthanized, with the pituitary and hypothalamus preserved for analysis. Antibody specificity was imperative for the IHC, in order to discriminate Kp from other RF-amide peptides. Therefore, the antibody selection was: 1°: rabbit anti- Kp and mouse anti-GnRH; 2°: Alexa fluor (594) goat anti-rabbit IgG and Alexa fluor (488) goat anti-mouse IgG. The mounted sections were evaluated with an epifluorescence microscope with wavelength filters for analysis of each fiber type (480nm - GnRH, green fluorescence; 560nm - Kp, red fluorescence).

Gonadotropin releasing hormone fibers were concentrated in the medial basal hypothalamus (MBH) with dense fibers and terminals throughout the median eminence (ME) extending into the arcuate nucleus (ARC). The Kp fibers had poorly defined boundaries and were scattered throughout the MBH and the lateral hypothalamic area, with clear demarcation within the lateral ARC. In contrast to the high frequency of GnRH fibers, Kp fibers were scarce within the ME. With regard to the morphological interactions between GnRH and Kp, the dual immunofluorescence illustrated very closely apposed Kp structures to GnRH neurons in the ARC of the caudal MBH. Characterization of the distribution of the GnRH and Kp fibers in the canine enables inferences regarding their neuro-endocrinological activity.



Photomicrographs: 1) GnRH - High fluorescence in the ME and lack of florescence in the pituitary (PT); 2 x magnification, 500 µm scale 2) GnRH - ME - dense organization and lack of fiber orientation; 20 x magnification, 50 µm scale 3) Kp - high fluorescence in the ARC and relative lack of fibers in the ventral aspect of the ME; 4 x magnification, 300 µm scale

Keywords: Kisspeptin, gonadotrophin releasing hormone, immunohistochemistry, hypothalamus, canine

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

Abortion in a mare due to umbilical torsion

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Normal foals have some degree of umbilical spiraling present at birth. Excessive torsion causes vessels to compress, causing associated edema and hemorrhage of the umbilical cord, which results in fetal death.[1-3] The etiology of severe umbilical torsions is unknown but thought to be related to increased cord length.[4] After death, fetuses often remain *in utero* long enough to undergo some degree of autolysis. Umbilical torsion is an important cause of abortion in mares.[5]

A six-year-old mare at 214 days gestation presented for signs of colic. Rupture of the chorioallanotic membrane and expulsion of fetal fluids was observed shortly after signs of colic were noted. Palpation of the fetus revealed a cranial longitudinal, dorsosacral presentation with neck and bilateral carpal flexion. Delivery was uncomplicated after malpositions were corrected with mutation. The umbilicus was noted to be severely twisted and swollen with areas of hemorrhage and edema along the length of the cord. Fetal membranes were incompletely expelled two hours later, with retention of the tip of the non-gravid horn. The mare was treated with flunixin meglumine, trimethoprim sulfadiazine, oxytocin, and uterine lavage. A small weight was attached via umbilical tape to the retained fetal remnant and it was expelled 12 hours later. Following an additional uterine lavage, treatments were discontinued. Necropsy of the fetus revealed severe autolysis and an umbilicus that was markedly torsed and edematous with areas of marked hemorrhage. Multifocal mineralization of microcotyledonary villi and allantoic blood vessels not associated with inflammation were signs consistent with umbilical torsion. There was no evidence of viral or bacterial infection.

Umbilical torsion is reported as the leading cause of abortion in the U.K.,[5] in contrast to fetoplacental infections in Kentucky.[6,7] This difference may be due to regional disease trends.[8]

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Case report: diagnosis of congenital short penis with a filling defect in a bull

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A 22-month old Brangus bull presented for infertility with a history of premature ejaculation and an inability to achieve intromission. A breeding soundness examination and three *Tritrichomonas foetus* cultures revealed the bull to be a satisfactory potential breeder and negative for trichomoniasis. However, the results of the test mating determined that intromission could not be achieved due to what appeared to be an inability to fully extend the penis. A contrast cavernosogram was performed which highlighted inadequate filling of the corpus cavernosum penis specifically involving the dorsal portion of the distal bend of the sigmoid flexure. The cavernous spaces proximal and distal to the defects were uniformly filled, and it was unclear if the filling defects were congenital or due to injury and fibrosis. Additionally, the length of the penis, even with full extension, was determined to be much shorter than that of a normal bull (90 cm), leading to the diagnosis of congenitally short penis with filling defects of the corpus cavernosum penis.

The consequences of this condition include the inability to naturally service cows. Bulls harboring these abnormalities should be removed from the breeding pool to prevent passing these traits on to offspring. The only possible use for the bull's semen would be utilization as a terminal sire in an artificial insemination program.

A congenitally short penis in a bull is an unusual finding with no reasonable treatment options. A breeding soundness examination only evaluates physical soundness and semen quality, but to be sure a bull is in fact a breeder, observation of intromission must be made. This case emphasizes that a breeding soundness examination alone does not deem a bull a satisfactory breeder, but additional test mating is warranted to fully assess the true breeding potential of the bull.

Keywords: Bull, short penis, cavernosography, breeding soundness examination

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Breeding soundness examination and semen cryopreservation in a dog with unilateral Sertoli cell tumor and perineal mast cell tumor

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A 9 year old intact male Old English Mastiff was referred to the Washington State University Veterinary Teaching Hospital for evaluation of a perineal mast cell tumor as well as breeding soundness examination (BSE) and semen cryopreservation. During the BSE a mass was detected via ultrasonography in the right testicle. Semen was collected and cryopreserved on two separate occasions prior to surgical removal of the perineal mass and both testicles. The initial progressive motility of the two ejaculates was 75% and 80%, respectively. The post-thaw motility was 45% and 50%, respectively. After castration, epididymal sperm were collected using the float-up technique and evaluated for motility, concentration, and morphology. The motility of extended semen from the left epididymis was 50% and the right epididymis was 0%. Concentration, determined via hemacytometer, and percent normal morphology were 34.5 million sperm/mL and 27% for the left epididymis and 4.0 million sperm/mL and 1% for the right epididymis, respectively. Histopathology confirmed the perineal mast cell tumor and diagnosed the testicular mass as a Sertoli cell tumor. Sertoli cell tumors affect variable ages and breeds, are rarely malignant, and are more common in cryptorchid testes.[1] They result in increased estrogen levels in up to 39% of cases.[1] No feminization or signs of a paraneoplastic syndrome were observed in this dog. This case highlights the importance of regularly scheduled BSE in all breeding males as the dog's overall semen analysis fell within normal parameters and did not demonstrate the pathology of the right testicle, which was only apparent upon ultrasonographic examination and comparison of the extragonadal sperm reserves.

Keywords: Canine, epididymal sperm, ultrasonography, neoplasia

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Hydrops allantois and amnion in a Thoroughbred mare

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Hydrops allantois and hydrops amnion are rare gestational complications of equine pregnancy.[1-3] Hydrops allantois is typically caused by placental dysfunction, while hydrops amnion is related to congenital abnormalities of the fetus.[3,4]

A 12 year old Thoroughbred mare presented to the University of Florida College of Veterinary Medicine on day 220 of gestation with signs of inappetance and possible placentitis. The mare's abdominal circumference was enlarged and circumference measurements were taken daily. The mare was also monitored with transrectal and transabdominal ultrasound. The combined thickness of the uterus and placenta and fetal heart rate were within normal limits. Fetal fluids had an increased echogenicity and the fetus was unable to be palpated transrectally. Three days after initial presentation no fetal heartbeat was detected. Abortion was induced with misoprostol and oxytocin. The chorioallantois was ruptured manually and approximately 100L of fetal fluids were released. The fetus was extracted and found to be a fetal monster. The placenta was retained and passed in its entirety 36 hours after induction. The placenta was grossly abnormal with large avillous regions. There were no gross signs of placental infection. Repeated transrectal ultrasound examinations revealed urine pooling and the development of a body wall hernia with no bowel entrapment. Necropsy findings indicated severe congenital malformations of the fetus, including severe hydrocephalus, palatoschisis, cranioschisis, bilateral anopthalmia, and severe lateral deviation of the muzzle.

Fetal malformations contribute to defects in fetal swallowing and processing of amniotic fluid, causing the excess accumulation of amniotic fluid.[3,4] Hydrops allantois was also diagnosed due to the large amount of fluid release at parturition, placental insufficiency, increased echogenicity of fetal fluids and the enlarged abdomen of the mare.[2,3] A breeding soundness examination was advised to assess endometrial abnormalities, urine pooling and resolution of the body wall hernia prior to rebreeding.[1]

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Equine oviduct dysfunction

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Although an uncommon cause of infertility, oviduct dysfunction should be considered when other etiologies are excluded. Pregnancy was never detected in a 5-year-old Standardbred mare despite good breeding management that included artificial inseminations with fresh semen from four fertile stallions during 12 estrous cycles over two breeding seasons. No genital tract abnormalities were apparent ultrasonographically and hysteroscopically and the mare had a normal karyotype. The mare was referred for a thorough examination of the genital tract and evaluation of the oviducts.

No significant abnormalities were detected on breeding soundness examination that included reproductive behavior evaluation, perineum assessment, palpation and ultrasonography per rectum, aerobic culture of an endometrial swab, histological evaluation of an endometrial biopsy sample, and visual and manual vaginal examination. Laparoscopic instrumentation for evaluation of the genital tract revealed a slight bulge at the junction of the ampulla and isthmus of the left oviduct. The ampulla and isthmus of the right oviduct was grossly distended and the mesosalpinx appeared inflamed with prominent blood vessels. Prostaglandin E_2 (0.25 mg; Prepidil Gel™, Pfizer Inc., New York, NY) was applied to the serosal surface of each oviduct to cause relaxation of the circular muscle of the oviduct and allow expulsion of oviductal accumulations.[1]

Ovulation occurred two days later on the right ovary. After cloprostenol (Estrumate™, Schering-Plough Animal Health, Union, NJ) administration, cooled transported semen was inseminated, and human chorionic gonadotropin was administered. Ovulation on the right ovary was detected 13 days after the oviduct procedure. Periodic palpation and ultrasonography per rectum revealed one conceptus appropriate for the stage of pregnancy.

Since oviduct dysfunction is challenging to evaluate, it is possible that oviductal pathologies exist more often than documented.[2-5] Oviductal dysfunction should be on the list of differentials in cases where the cause of the infertility has not been determined.

Keywords: Horse, mare, oviduct, PGE_2 gel, laparoscope

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Successful nonsurgical management of uterine torsion in the mare

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Uterine torsion, the relatively uncommon pathologic rotation of the uterus along its long axis,[1] occurs most commonly from late gestation until term in the mare.[2] Standing flank laparotomy or ventral midline celiotomy are the most commonly accepted methods of treatment for equine uterine torsion. Rolling of the anesthetized mare is the seldom used nonsurgical approach which has been shown to increase rate of survival for both mare and foal.[3]

A 15-year old Thoroughbred mare was referred for colic symptoms and impending abortion (280 days gestation). Transabdominal ultrasound revealed an abnormal location of the fetus and a fetal heart rate of 88 bpm. The left broad ligament was displaced to the right and dilated vessels were identified near the cervix (color flow Doppler), consistent with a right uterine torsion. After induction of general anesthesia, the mare was placed in right lateral recumbency with a wooden plank positioned in the flank to stabilize the fetus during rolling. The mare was rolled twice to correct the torsion. Position of the broad ligaments was assessed after each rolling. Transabdominal and transrectal ultrasounds were performed to assess fetal well-being, combined thickness of the uterus and placenta, and areas of separation, which were all within normal limits. The day following admission, the mare was discharged from the hospital. A healthy filly was delivered at 340 days of gestation.

Rolling of the mare is an acceptable alternative to surgery, provided that the direction of the torsion can be definitively determined, there is no gastrointestinal tract entrapment, the degree of rotation is not severe, and the mare is not at term. Nonsurgical treatment does not allow examination for gastrointestinal involvement, but eliminates the occurrence of post-operative complications for the mare and fetus and is more economically feasible, offering an effective non-surgical alternative for treatment of uterine torsion.

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Investigation of estrus induction failures following Ovuplant® administration in dogs

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Introduction

The deslorelin implant, Ovuplant® (Ayerst Laboratories), is licensed for use in horses and has been shown to reliably induce estrus in the majority of anestrous bitches.[1,2] Anovulation and abortion due to premature luteolysis have been reported during the induced estrus and subsequent luteal period, respectively. The objective of this study was to evaluate the endocrine response in a diverse population of privately owned bitches that did not become pregnant after receiving an Ovuplant® for estrus induction. We hypothesized that ovulation failure, diagnosed by serum progesterone concentrations (P4) that failed to rise above 5 ng/mL was the most common cause of reproductive failure.

Keywords: Canine; deslorelin; estrus induction; GnRH; ovulation failure

Methods

Privately-owned bitches (n=37) were presented for Ovuplant® estrus induction and breeding management. Some of the bitches were affected by cystic endometrial hyperplasia before estrus induction was attempted. Ovuplant® implants were placed in the vestibular submucosa during anestrus, at least four months after the last estrus and when P4≤1 ng/mL. Serial venous blood samples for P4 determination were collected to determine the optimal time for breeding. Progesterone was measured using the Immulite chemiluminescent immunoassay system (Siemens Diagnostics). Bitches were bred, naturally or artificially, one to three times to a male selected by their owners. Implants were removed within three days of the last breeding or within 21 days for bitches that did not ovulate.

Results

Of the 37 bitches, 16 (43%) did not become pregnant, including seven (19%) that did not ovulate (P4≤3 ng/mL). The remaining nine bitches displayed P4 profiles that were not different from those published for spontaneously cycling bitches.[3] Two bitches that did not ovulate and one bitch that ovulated but did not become pregnant failed to show any external signs of pro-estrus. None of the pregnant bitches aborted. Following the Ovuplant® induced cycle, 13 of the 16 bitches were bred during a subsequent spontaneous estrus, and of these 10 became pregnant. Three bitches were not re-bred.

Conclusion

Ovuplant® treatment is an effective method for inducing estrus, but ovulation failure must be expected in a significant number of treated bitches.

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The use of a canine GnRH vaccine for behavior modification in cats

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Introduction

It is estimated that four to five million cats enter U.S. shelters each year, and more than half of these animals are euthanized because there are not enough homes for them or because of behavioral reasons that result in relinquishment. Inappropriate elimination and aggression towards other cats in the household are two reasons given for relinquishment.[1] We hypothesized that persistently elevated luteinizing hormone (LH) concentrations after gonadectomy are responsible for these behaviors. The objective of this study was to determine the safety and efficacy of a canine gonadotropin releasing hormone (GnRH) vaccine for the purposes of LH suppression and behavior modification.

Keywords: Aggressive behavior; cat; GnRH; inappropriate urination; vaccine

Methods

Three 5-year-old, privately-owned male neutered cats (two domestic short-haired (DSH) cats, one Bengal cat) were used for this study. The GnRH vaccine (Canine Gonadotropin Releasing Factor Immunotherapeutic®; Pfizer Animal Health) was administered twice by subcutaneous injection eight weeks apart. Vaccination sites were inspected and palpated for swelling by the owners of the cats for one week after vaccination. Venous blood samples (2 mL) were collected prior to each immunization and every eight weeks thereafter for six months. Semi-quantitative LH concentrations were determined using the WITNESS® LH test kit (Synbiotics, Kansas City, MO), which is commonly used for diagnosing the presence or absence of retained ovarian tissue in queens. Antibodies against GnRH were determined using an ELISA and titers were compared using ANOVA (Stata V.12, Statacorp, College Station, TX). Significance was defined as $p<0.05$.

Results

Following vaccination, cats showed mild discomfort at the injection site. This was prevented with an injection of carprofen administered at the time of the booster injection. No other side effects were reported. The two DSH cats displayed decreased frequency of inappropriate urination following the second vaccination. However, the aggressive behavior displayed by the Bengal cat towards the other household cats did not subside following vaccination. All cats had a positive LH test result (LH ≥ 1ng/mL) at 0 and 8 weeks, which became negative (LH < 1 ng/mL) at 16 and 24 weeks. All developed GnRH antibody titers, which were significantly higher at weeks 8 and 16 compared to weeks 0 and 24.

Conclusion

These results indicate that a GnRH vaccine labeled for the treatment of benign prostatic hyperplasia in dogs is probably safe in cats and may be effective in treating inappropriate urination in adult male neutered cats.

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Bacterial growth and semen viability in canine semen extenders inoculated with *Brucella canis*

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Brucella canis, the etiological agent of canine brucellosis, was first described in 1967 by Leland Carmicheal. Since that time several serologic tests have been developed to diagnose this disease in domestic canids. The surface antigens of this bacteria make many of serologic tests highly sensitive, but lacking in specificity. In addition to this, there is a substantial lag time between the initial exposure and infection to seroconversion or positive blood culture, during which these dogs can be contagious. Due to the dilemma of accurately diagnosing canine brucellosis in a timely manner, it is difficult to determine if the dogs and semen that the practitioners and owners are handling during semen collection, processing, shipment and artificial insemination are truly disease-free in the face of negative serologic test results. The purpose of this experiment was twofold: 1) determine if commercial semen extenders will inhibit the growth of *Brucella canis* and 2) in the event that commercial semen extenders do not prevent the growth of *Brucella canis*, determine if the addition of antibiotics to the semen extenders inhibit the growth. It was anticipated that all of the commercial extenders, with the exception of the Kenney skim milk extender would prevent the growth of *Brucella canis*. In this experiment, six commercially available extenders (Kenney skim milk extender without antibiotics (Veterinary Concepts, Spring Valley, WI), Fresh Express® (Synbiotics, Kansas City, MO), CLONE™ (CLONE Inc., Doylestown, PA), CaniPRO AI and CaniPRO Chill 5™(Minitube of America, Verona, WI), Insemin-aid™(Camelot Farms, College Station, TX) were examined. In experiment 1, 20µL of a 0.5 McFarland standard *Brucella canis* suspension was added to 1 mL aliquots of each extender, a control saline, and stored at 5°C. Brucella blood agar plates were inoculated with 100 µL (approximately $2x10^5$ CFU) of each suspension at 0, 24, 48, and 120 h of chilled storage. All suspensions were plated in duplicate and incubated at 37°C for up to 72 h. None of the extenders inhibited the growth of *Brucella canis*. In experiment 2, three antibiotics (amikacin, 4 µg/mL; ampicillin, 2 µg/mL; ticarcillin, 8µg/mL) were added to each extender-*B. canis* suspension. None of the extenders with supplemental antibiotics inhibited the growth of *Brucella canis*, but all of the extenders with the exception of CLONE™ had fewer colony forming units (CFU) than those without supplemental antibiotics. With this information, semen from two stud dogs was added to the extender/*B. canis*/antibiotic mixtures at a ratio of 1:5 (semen : mixture). The addition of semen led to an increase in CFUs of *Brucella canis* in all extenders. The information gained in this study reveals that the use of commercial semen extenders with and without the addition of antibiotics does not inhibit the growth of *Brucella canis*.

Keywords: *Brucella canis*, canine brucellosis, semen extenders, bacterial growth, dog

Pre-scrotal vasectomy in alpacas (*Vicugna pacos*) – Technique and complication rate

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Vasectomy is a technique used in research models which investigate seminal plasma, as the procedure removes contributions of the testes and epididymides. Application of ruminant vasectomy techniques in alpacas is complicated by the non-pendulous scrotum. Laparoscopic techniques require specialized equipment. The objective of this study was to evaluate the efficacy and complications of a pre-scrotal vasectomy technique in male alpacas. Twenty-two males aged 2-7 years were submitted for semen collection by electroejaculation (EE) under general anesthesia, which was induced with an intramuscular injection of ketamine 4.0 mg/kg, xylazine 0.4 mg/kg, and butorphanol 0.04 mg/kg and maintained with isoflurane in oxygen via endotracheal tube. All males produced ejaculates containing spermatozoa. Long-acting ceftiofur (Excede®, Pfizer Animal Health, New York, NY) 6.6 mg/kg SQ and flunixin meglumine 1.1 mg/kg SQ were administered prior to vasectomy. Surgery was performed in dorsal recumbency. The inguinal region was clipped and surgically prepared. The spermatic cord was palpated lateral to the prepuce, caudal to the inguinal ring. A 2 cm skin incision was made over the spermatic cord and the vaginal tunic opened with a stab incision. The ductus deferens was isolated with hemostats and a 2 cm segment was identified. Two ligatures of 2-0 polydioxanone were placed around the segment and it was incised using Metzenbaum scissors and removed. The tunic was left open and the skin was closed using 2-0 polydioxanone in a subcuticular pattern. Animals were monitored for complications. Of 8 animals (Group 1) operated on by clinicians, no complications were noted. Of 14 animals (Group 2) operated on by veterinary students under clinician supervision, two males developed diarrhea post-operatively, which resolved in one animal in two days. The second animal developed a fever which was treated with flunixin meglumine. The animal suffered severe weight loss but the clinical signs had resolved by five weeks post-vasectomy. The exact cause of the diarrhea was not determined. No local reactions or complications were observed at the incision sites. Success of vasectomy was verified by EE under general anesthesia five weeks post-vasectomy. Three animals from Group 1 had a few dead spermatozoa in their ejaculates. In Group 2, one animal had a normal ejaculate with viable spermatozoa suggesting failure of vasectomy. The overall success of vasectomy was 95.5% (21/22). Based on these results, pre-scrotal vasectomy appears to be a safe and reliable method of vasectomy in alpacas. The chances of fertilization are practically nil five weeks post-surgery. The removed segments of vas deferens should be submitted for histologic confirmation.

Keywords: Semen; electroejaculation; surgery; camelid; reproduction

Effects of a second freeze-thaw cycle on bighorn sheep (*Ovis canadensis canadensis)* semen motility and membrane integrity

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Only a fraction of a dose of frozen semen is needed when advanced reproductive biotechnologies are used. The ability to refreeze thawed semen would be a valuable tool to save germplasm from rare or endangered species. Big horn sheep are a threatened species that may benefit from these technologies. We hypothesized that refreezing will allow preservation of viability of a significant proportion of previously frozen-thawed big horn epididymal spermatozoa. The objective of this experiment was to study the effect of a second freeze-thaw cycle on big horn sheep epididymal sperm motility and membrane integrity.

Epididymal sperm were previously harvested and frozen from two big horn rams that died from pneumonia.[1] Semen from each ram (n=8 replicates per ram, 2 x 0.5 mL per replicate) was thawed in a water bath (37°C) for 30 seconds. Aliquots were taken from each replicate and evaluated for progressive motility, membrane integrity, and acrosome integrity. Motility was evaluated subjectively (400x). Membrane integrity was evaluated by hypoosmotic swelling test (HOST) (10 µL of sperm incubated in 190 µL of 100 mOsm sucrose solution).[2] Acrosome integrity was evaluated using the Spermac® (Minitube, Verona, WI) staining technique.[3] Hypoosmotic swelling test and Spermac® evaluations were performed by evaluating 100 spermatozoa from each sample. The remaining frozen-thawed semen was repackaged in 0.5 mL straws and frozen in liquid nitrogen vapor as described previously.[1] Refrozen semen was thawed and evaluated one week later in the same manner as described above.

Data were analyzed by ANOVA. There was no significant ram effect; therefore the data were analyzed with refreezing as the main treatment. As expected, all sperm quality parameters were significantly lower after refreezing. Post-thaw sperm quality after refreezing decreased on average by 48%, 38%, and 28%, for acrosome integrity, progressive motility, and membrane integrity, respectively. The discrepancy observed in the quality of the sperm as assessed by HOST and Spermac® merits further evaluation. This study shows that refreezing of big horn epididymal sperm results in recovery of at least 50% of the initial spermatozoa. Further studies are in progress to optimize the refreezing process using different refreezing rates and to evaluate in vivo fertility of refrozen-thawed semen.

Keywords: Sperm, fertility, refreeze, cryopreservation, endangered species

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A rare case of persistent testicular infection causes shedding of infectious virus in semen

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Recently, a dairy bull in the United States was diagnosed as the second confirmed case of persistent testicular infection with bovine viral diarrhea virus (BVDV). This clinical case report characterizes the bull's infection, humoral immune response, and epidemiologic significance.

Virus neutralization assays were performed to detect anti-BVDV antibodies in serum. To detect BVDV, (1) virus isolation, antigen capture ELISA, and various PCR assays were performed using serum; (2) PCR assays were performed using whole blood samples; and (3) direct immunoperoxidase staining, PCR, titration with subsequent immunoperoxidase monolayer assay and passage with subsequent virus isolation were performed using cryopreserved semen. Dual and serial immunohistochemical staining was performed to detect BVDV in germ and Sertoli cells, respectively, within testicular biopsies obtained at 33 months of age. Centrifugal separation with PCR assay of the supernatant and cell pellet was performed to determine if virus was cell associated and/or detectable free in seminal plasma. Sequencing of 248 nucleotides was performed to determine viral subgenotype. Epidemiologic investigation involved sequential virus neutralization assays of serum obtained from bulls and steers before, during, and after contact with the infected bull.

Between 6 and 24 months of age, this bull lacked BVDV in seven sequential serum samples and two peripheral white blood cell samples. The bull was seropositive to type 1, BVDV strains with serologic endpoints of 256, 2048, and 4096 at 8, 19, and 22 months of age, respectively. At 24 and 29 months of age, the bull exhibited serum neutralizing antibody titers of 4096 and 16384, respectively, to the strain isolated from his semen. The bull produced 25 collections of semen from 14 to 22 months of age that consistently contained BVDV as determined by direct immunoperoxidase staining, PCR and virus isolation when semen was shipped to the laboratory in a liquid nitrogen dry shipper. The concentration of infectious virus in semen ranged from < 250 to 6250 $CCID_{50}$/mL with a median of 1250 $CCID_{50}$/mL. Virus was detected in association with Sertoli and germ cells within some seminiferous tubules. Virus was not detected free in seminal plasma but was readily detected in association with pelleted cells. Sequencing revealed a 1a subgenotype of BVDV. Virus was not transmitted to directly contacted bulls and steers.

In conclusion, natural exposure to a 1a strain of BVDV can cause persistent testicular infection of at least nineteen months duration. This infection of a seropositive, non-viremic bull can cause contamination of semen with infectious virus.

Keywords: Bovine viral diarrhea virus, persistent testicular infection, semen

Presence of bacteria in the reproductive tract of healthy stallions and its relation to the fertility of mares

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Colonization of bacteria in the reproductive tract of both stallions and mares can cause numerous fertility issues. Breeding operations that only utilize natural cover breeding are faced with a difficult challenge to control disease outbreaks of pathogenic bacteria. The current study used Thoroughbred stallions and mares from central Kentucky to (1) investigate the occurrence of potentially pathogenic bacteria on the stallion's external genitalia based on cultures, (2) determine if there is an impact on pregnancy rates and pregnancy loss when a stallion has a positive culture and continues to breed, and (3) investigate the occurrence of bacteria and type of isolate in the mare's uterus after breeding by live cover to stallions with or without positive bacterial cultures.

This study utilized 15 Thoroughbred stallions and 206 mares from two central Kentucky farms during the 2010 and 2011 breeding seasons (selection criteria for stallions: book size >20 mares, normal fertility, and all mares bred by live cover). Samples for bacteriological evaluation were taken from the prepuce and the urethra after ejaculation (n=201) of stallions. Uterine swabs (n=264) were collected 18-36 hr after breeding and farm records were utilized to track pregnancy rates at day 14 and pregnancy losses (any loss of pregnancy after day 14). Statistical analyses were performed using SAS software (SAS Institute, INC., Cary, NC). The GLIMMIX procedure was used to test the effect between stallion culture results with pregnancy rates and pregnancy loss in mares. Random effects were the farm and stallion. Fixed effects were stallion culture results, beginning status of the mare, number of times the mare had been bred, and year. Chi-square was used to test the effect between stallion culture results and post-breeding mare uterine culture results, the effect between bacterial types found on the stallion cultures and pregnancy rates and pregnancy loss, and the effect between bacterial types found on the stallion cultures and bacterial types found on the post-breeding uterine cultures.

Of stallion cultures, 22.4% were positive for potentially pathogenic bacteria, with *S. zooepidemicus* (51.1%) being the most common. There was no difference in pregnancy rates at day 14 and pregnancy losses between stallions negative or positive for potentially pathogenic bacteria. Lastly, 29.2% of the uterine cultures were positive for potentially pathogenic bacteria, with *S. zooepidemicus* (90.9%) being the most common. There was no difference in the occurrence of bacteria or type of isolate found on uterine cultures after breeding stallions with or without positive cultures. In conclusion, the occurrence of potentially pathogenic bacteria on the stallion's external genitalia did not affect fertility of stallions or mares.

Keywords: Stallion, external genitalia, bacterial flora, uterus, pathogenic bacteria

Effect of antibiotic treatment of mares prior to transvaginal follicle aspiration on embryo development after in vitro oocyte maturation and intracytoplasmic sperm injection

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Transvaginal follicle aspiration (TVA) is commonly utilized to obtain oocytes from live mares for assisted fertilization techniques, such as intracytoplasmic sperm injection (ICSI). Ovarian abscess formation after multiple TVAs has been reported, although the incidence is low. Prophylactic administration of antibiotics before follicle aspiration might minimize the possibility of ovarian infection; however, since oocytes are exposed to frank blood during the aspiration procedure, our hypothesis was that systemic antibiotics may decrease the viability of aspirated oocytes. This study examined embryo development after ICSI of immature oocytes recovered from mares treated with systemic antibiotics before the TVA procedure. The study was done in December through February, to provide information for the next breeding season. Oocytes were recovered from all visible follicles of 10 mares either treated with ampicillin (2 mg/kg, IV) and gentamicin (6.6 mg/kg, IV) one time, within 10 minutes of the start of aspiration, or left untreated. Recovered oocytes were held in 40% M199 with Earle's salts, 40% M199 with Hanks' salts, and 20% FBS overnight[1] and were then cultured in maturation medium (M199 with 10% fetal bovine serum and 5 mU/ml FSH) for 30 h. Oocytes having a visible polar body were injected with spermatozoa via Piezo drill, and presumptive embryos were examined for cleavage at Day 5 and for blastocyst development from Day 7 through Day 11. Aspiration was not performed unless >5 follicles ≥8 mm diameter were present. Mares were crossed over to the alternative treatment group after the first aspiration. A total of 16 aspirations were performed, seven in the control group (100 follicles) and nine in the antibiotic-treated group (128 follicles). There was no difference ($P > 0.1$; Fishers exact test) between the control and antibiotic-treated groups in recovery rate (59% and 66%, respectively); oocyte maturation rate (59% and 60%, respectively); cleavage rate (79% and 86%, respectively); or blastocyst rate (18% and 18%, respectively). These results indicate that systemic administration of antibiotics before TVA does not reduce the rates of maturation or embryo development of recovered oocytes. Oocytes recovered from immature follicles during the non-breeding season were capable of blastocyst development after ICSI.

Keywords: Equine, transvaginal aspiration, oocytes, antibiotics, intracytoplasmic sperm injection

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Progestin treatment of preovulatory mares fails to delay ovulation and may impair fertility

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Controlling ovulation to occur when popular stallions or transported semen is available is desirable to effectively manage broodmares. Some strategies successfully hasten ovulation (e.g., human chorionic gonadotropin and deslorelin), but there is no effective method to delay ovulation in mares. Our hypothesis was that progestins administered during the preovulatory period would delay ovulation without interfering with fertility. The objectives of this study were to determine if: i) CIDR-B or altrenogest can delay ovulation; ii) spontaneous ovulation follows cessation of treatment; iv) treatment affects fertility; v) treatment affects endometrial edema; vi) follicular growth and estrus is suppressed. Fourteen cyclic reproductively sound mares were examined by per rectal palpation and ultrasonography, every one to three d, according to ovarian and uterine findings. Once mares were confirmed in estrus with a large pre-ovulatory follicle ($\geq$ 35 mm), they were sequentially and randomly allocated in a cross-over design, to one of three treatments: 1) control (no treatment, n = 25 cycles); 2) altrenogest (Regu-Mate® 0.044 mg/kg/d, for 48 h, n = 17 cycles); and 3) CIDR-B applied intra-vaginally for 48 h (n = 16 cycles) and were teased daily with a mature stallion until ovulation was detected. Mares were bred with cooled extended semen from a single fertile stallion within 24 h of semen collection, starting two d after treatment onset; mating was repeated every 48 h until ovulation was detected. Pregnancy diagnosis was performed 12 to 14 d after ovulation. Once confirmed pregnant, the embryonic vesicle was manually reduced and the mare received prostaglandin to return to estrus. All mares were used for four to six cycles, allowing each treatment to be repeated one or two times. The data were analyzed using StataIC® 10.0 (College Station, TX). The pregnancy rate (dichotomous outcome) was evaluated by multiple logistic regression, controlling for mare (random variable), cycle number, treatment and artificial inseminations (AIs) per cycle (fixed variables). Mixed linear model (continuous outcomes) was used to analyze follicle growth rate, time to ovulation, endometrial edema and estrus behavior, using the same random and fixed variables (except AIs per cycle). Neither CIDR nor altrenogest treatment delayed ovulation. The preovulatory follicular diameter was not affected by treatment. Treatment had no effect on rate of follicular growth and there were no significant differences in slope (P = 0.51) or intercept (P = 0.85) of the lines for follicle diameter relative to day of ovulation when corrected for day of ovulation. There were no significant differences between altrenogest, CIDR, and control groups for the number of matings per cycle (P = 0.27). Both treatments had prompt and dramatic effects on abolishing estrus behavior within 24 h (P < 0.0001); behavior returned to the control level after cessation of treatment. Both forms of treatment reduced endometrial edema by 24 h (CIDR) and 48 h (altrenogest); the edema score returned to normal after cessation of treatment within 24 h. Altrenogest treatment tended to reduce the pregnancy rate (P = 0.09). In summary, altrenogest or CIDR treatment of mares with preovulatory follicles did not delay ovulation, retard follicular growth, or reduce ovulatory diameter. Therefore, the use of progestins to delay ovulation in mares should be discouraged, because it lacks efficacy and may reduce successful establishment of pregnancy.

Keywords: Fertility, ovulation control, estrous behavior, mare.

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Unilateral benign cystadenoma in a broodmare

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Significance

This following case demonstrates continued reproductive success in a broodmare with long-term benign unilateral ovary enlargement.

Case report

A 23-year-old Quarter Horse mare was presented for unilateral ovarian enlargement discovered during routine breeding management. The left ovary, cervix, uterine body and uterine horns were normal on transrectal palpation and ultrasonography, and there was evidence of cyclicity. The right ovary was grossly enlarged and ventrally displaced. The ovary had an irregular round contour and a rough surface. The ovulation fossa could not be palpated. Using transabdominal ultrasonography through the flank, the entire right ovary could be imaged. Multiple 20-80 mm anechoic fluid-filled structures were present throughout the entire ovary. Normal ovarian parenchyma could not be visualized. Serum estradiol, testosterone and inhibin concentrations were all within normal limits. Based upon its size, ultrasonographic characteristics, and the absence of an endocrine contribution, the differential diagnoses were cystadenoma, germinal inclusion cysts, cystic rete ovarii, or cystic degeneration of other areas of the intra-ovarian mesonephric tubules (e.g. cystic epoophoron or paroophoron). Removal of the mass by ventral midline laparotomy was recommended because the ovary size prevented any other surgical approach. Despite the guarded prognosis for failure to removal, the owner declined surgery due to cost. The mare was subsequently bred over the next five breeding seasons and delivered four healthy foals.

Follow up

Two years following delivery of her last foal, the mare re-presented for significant weight loss and hepatopathy was diagnosed on the basis of elevated liver enzymes. Ultrasonography of the right ovarian mass showed no change in size or echotexture. Euthanasia was elected by the owner at this time. Necropsy revealed a cirrhotic liver, severe cardiomegaly, and hemoabdomen that were independent of the enlarged ovary. The right ovary was 18 cm in diameter with multiple cystic structures and fibrous tags on the ovary surface. Histologically, the epithelial lining was very uniform with columnar epithelial cells that were occasionally ciliated. There was no evidence of cellular atypia and the mitotic index was very low. The stroma contained compressed ovarian structures. Based upon these findings, a definitive diagnosis of benign cystadenoma arising from either the ovulation fossa or fimbria was made. In a previous report on an equine ovarian cystadenoma,[1] plasma testosterone concentrations were high at the time of examination, but decreased to normal values after removal of the cystadenoma. This endocrine involvement differed from the present case because despite multiple evaluations over the course of seven years, serum estradiol, testosterone and inhibin concentrations always remained within normal limits.

Keywords: Cystadenoma; equine; ovarian tumor; ultrasonography

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Administration of ceftiofur crystalline free acid (CCFA) to pony mares with placentitis

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Ascending bacterial equine placentitis initiates inflammation, uterine contractions and preterm delivery. Treatment protocols for placentitis have included antimicrobials, anti-inflammatory agents and progestins. In 2010, a long-acting preparation of ceftiofur crystalline free acid (CCFA) with broad-spectrum bactericidal activity was approved for use in horses. Our objectives were to determine the pharmacokinetics of CCFA in pregnant mares with placentitis, evaluate the disposition of CCFA in fetal fluids, fetal membranes, colostrum and serum from foals, and to obtain pilot data on the efficacy of CCFA for improving foal survival in mares with induced placentitis. We hypothesized that administration of CCFA to mares with placentitis would result in therapeutic concentrations of desfuroylceftiofur acetamide (DCA, the acetamide derivative of ceftiofur) in tissues and fluids of mares and foals. Twelve reproductively normal, pregnant pony mares were assigned to one of three groups: CEFT (n = 3; CCFA 6.6 mg/kg, IM, q96h; Excede® Pfizer Animal Health, Kalamazoo, MI); COMBO (n = 6; CCFA 6.6 mg/kg, IM, q96h; altrenogest, 0.088 mg/kg, PO, q24h; pentoxifylline 8.5 mg/kg, PO, q12h); UNTREAT (n = 3; infected, untreated controls). From March through May in 2010, each mare was inoculated intracervically with 10^7 CFU *S. zooepidemicus* on day 286 of gestation (range 280-294). Treatment began at the onset of clinical signs (ultrasonographic evidence of increased combined thickness of uterus and placenta (CTUP), placental separation, mammary gland development, or vulvar discharge). Concentrations of DCA were measured in multiple tissue and fluid compartments from mares and foals. Serum DCA concentration versus time data was analyzed by noncompartmental pharmacokinetics. Significance was assigned for values $P < 0.05$. The serum half-life of DCA in pregnant mares with placentitis was 56.5 ± 20.5 h. Maximum and minimum serum concentrations of DCA at steady state in treated mares were 2.40 ± 0.40 μg/mL and 1.06 ± 0.29 μg/mL, respectively, which is similar to that in non-pregnant horses. Concentration of DCA in colostrum was 1.51 ± 0.60 μg/mL. Concentrations of DCA in placental and fetal tissues (median = 0.03 μg/mL) were below the minimum inhibitory concentration of relevant pathogens (0.2 μg/mL); DCA was not detected in amniotic fluid or serum from live foals. Treatment did not improve foal survival (Group CEFT: 0/3; Group COMBO: 2/6; Group UNTREAT: 2/3). Bacteria were recovered from the uterus of most mares and fetal/neonatal blood samples regardless of treatment group. The survival data were complicated by the unexpected number of live foals from infected, untreated mares (attributed to technical failure when placing bacteria in the mare's cervix). The results of this study do not support the use of CCFA at the recommended dose for the treatment of placentitis in mares.

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