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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.

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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

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Case Presentation. Presenting features, pertinent medical history, herd history (if applicable)
Differential Diagnosis. (if relevant)
Treatment.
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Manuscripts not received in time to be included in the September 2017 issue of Clinical Theriogenology will be published in the December 2017 issue.

The 2017 Bartlett Address
Ramblings of an idle warped mind

Clifford F. Shipley
College of Veterinary Medicine, University of Illinois, Urbana IL

I got a telephone call in February from Mike Thompson while on my way to Minnesota to speak. First thought….why is Mike calling me. Did they finally figure out that I was incompetent and wanted to take my Diplomate status away? Ban me from ACT/SFT? Draw and quarter me?

No, he wanted to congratulate me for being the David E. Bartlett Award for Lifetime Achievement in Theriogenology! I was speechless (hard to do). Honored. Speechless. Honored. Did I just outlive everybody else who was actually qualified? I know I did not pay anyone. Maybe they got nominations mixed up. Regardless, I am here. Honored! I am still struggling to figure out why. I just did my job. Excellence was my standard. Never left a call not wondering what else could I have done. Probably close to three thousand students (and counting) and always wondering if I could have explained it better, presented it better, or just been better. I know I did not get this award for my research efforts. I am clinical but hopefully scientific enough that I know some will get better even if I do not treat them or in spite of my treatment.

I have many people to thank for this award. First, my wife, Victoria A. Rowe, PhD for which I may never have started down this path. She decided that my mixed practice career in a small Iowa town might not be perfect for both of us! After updating my resume and writing a cover letter, she allowed me to sign it and send it off to Virginia-Maryland Regional College of Veterinary Medicine. I had carefully explained to her that universities wanted advanced degrees and boarded people. Unfortunately, they offered me a job and I had to eat crow. There I got to work with great theriogenologists like Beverly Purswell, Bill Ley, Dave Sprecher, and Jim Bowen while doing ambulatory and extension (by the way, what is a Hokie?)

Then it was back to Iowa for some corporate work with ASDIC Ltd and Yoder Feeds, which kept us clothed, fed, and me on the road. We were blessed with two wonderful children, Clinton Ryan and Abriel Jeane. Clint was a "bug and dinosaur" kid and Abby was our beautiful "computer" daughter. Family kept Vicki busy and made me realize that being on the road was not going to be conducive to a good family life. Lesson one… family first! So off to job hunting and the University of Illinois I went. There, I was introduced to the likes of Ted Lock, Randall Ott, Bruce Brodie, Howard Whitmore, Bill Wagner and a host of great residents and interns over the years. Others that have come and gone from Illinois are Sherrie Clark, Jim Brendenmuhl, Gary Althouse, Manoel Tamassia, Swanand Sathe, Katie Naughton, Hilary French, Deb Sauberli, Jacque Fusilier and others that my mind will think of later. Today I mostly push paper, limp, and work with Drs. Canisso, Lima, Ellerbrock and Herrmann. Illinois worked out well for family and career. I studied and took the practitioner route to ACT boards. Work all day, home with the kids and then study after they went to bed. Lesson 2…do not make the practitioner route so untenable as to limit/prohibit people from trying.

I would also like to thank my mother and father, Malcolm and Emma Jeanne Shipley, the finest two parents a child could have. Dad was a perfect role model. A WWII vet and 90% disabled, he never complained and worked hard every day. Up at five, do chores, breakfast at seven, and work all day in the field or with the livestock and then chores at five and supper at six. If there happened to be more daylight, it was back to work until dark. Once Randy Ott asked who my hero was and I replied that my Dad was. Still is. He passed at eighty-five years with shrapnel in his hands that could not be removed. Mom is still with us today. Eighty-seven and still full of wisdom and kindness. On the refrigerator door, windows, walls and other places reside her little clippings and writings of inspiration and knowledge. She raised four boys and lost two others. She could outrun me until I was twelve and kept the paddle on top of the refrigerator. Probably saved my life by keeping me in line. They "allowed" me all my pets and livestock. From pigs, sheep, cows, horses, rabbits, ducks, geese, turtles and on, they nurtured my mind and spirit. Piano lessons were mandatory, as was church, Methodist Youth Fellowship, band, Cub Scouts and lots of playing in the "crick" (creek for you non-southern Iowans). We were poor, but never wanted

for anything. A large garden that if you were bored, you were allowed to go weed. Lesson three…you do not have to have money to be rich!

Others who have influenced me along the way would be the first veterinarian that I remember. Dr. Pollack of Villisca, Iowa. I was probably six when he came to treat a down cow with milk fever. A true miracle to an unsuspecting lad. Always wanted to be a veterinarian. Still do. If I could figure out a way to pay the bills, I would do this for nothing. Lesson four…do things that bring you joy and make work seem like fun! The next most important veterinarian was Dr. Richard Riese. I was still in high school and he let the poor kid from Nodaway help him work cattle, spay dogs and clean the chute! He later returned to Iowa State University and did a residency and I had the pleasure of being one of his students (again). Others at ISU that influenced me were Steve Hopkins, Tracy Clark and Larry Evans. Dr. Clark was very protective of the repro mares so you would find Dr. Hopkins or Dr. Evans and ask them if you could palpate "extra"! He also did not appreciate riding the mares down the hall, but….Lesson five…it is not what you know it is who you know.

Now, to be serious for a very brief time. I see an erosion of theriogenology's place in academia (in Illinois for sure) or we would not be talking about it repeatedly. I do not have a solution. We (veterinarians) have become burdened with two paradigms. Production versus pets (human medicine). That is hard to reconcile some days. One of my early non-therio colleagues at Illinois once declared to me that all the profit in hog production is made in the finisher. I reminded him that nothing happens in the finisher unless something happens in the breeding barn. Lesson six… all production begins with reproduction. Lesson seven…even pets start with reproduction!

I used to and still do tease my equine brethren when doing colic surgery or some other super-duper complicated thing to something that is still going to die, that "don't worry, I'll make another one"! Lesson eight…we are/they are "still gonna die". At what cost do we practice? Real medicine? Standard of practice? Are we as veterinarians going to pass the lifeboat test? At what point do we lose our perspective? I have no problem providing the best of care, but also do not belabor that many do not want, need or have the resources for that kind of care. A very smart resident (Julie Funk) once told me that people come to us for options. Dr. Ott drilled the students that the probability of a favorable outcome was paramount to the treatment decision. Lesson nine…there are always two treatment options at least…do nothing and euthanasia. In between those two are the Ford, Chevy, Cadillac and Mercedes options.

Almost done. Lifelong learning is one the most important duties that we have to our clients and patients and to ourselves. Attend meetings. Read proceedings. Read books. Read newspapers. Anything! My mother said education is the one thing they cannot take away from you. Lesson 10…keep learning….never say no to an opportunity to learn. Even if it is going to be a hard lesson! That is probably what led me down this road. Eat crow while it is warm and stop digging when you find yourself in a hole. Leave plenty of dead behind. That means you have done something. Be it a lion, tiger or bear, just do it.

Last, thank you goes to the SFT and the ACT. You have become my brothers and sisters and are collectively, the finest people to be associated with that I have ever come across. I would ask that all read the first Bartlett Award talk by the Dr. Dave Bartlett. We owe everything to our founders and the battles that they fought for us. History is important. How we got here is important. What we leave behind is even more important.

Now to end with a Dr. Shipley story with a moral at the end. I was working on the Navajo Indian Reservation back in the early 1980's volunteering. Great fun, adventure, new place, challenges! A colic was coming in and I drew the short straw. While waiting for the horse to arrive, I went over everything that a senior veterinary student should know about colic. The professors and books had never been to the reservation. Up drove an old pickup truck with the cab jammed full of people and the bed of the truck full as well (I think thirteen people in all). Pulling an old two-horse trailer that was rusted and carrying a two-year-old palomino stallion, body condition score one. Par for the reservation. I tried to carry on a conversation with the owner, great grandpa, but he spoke little or no English. I worked my way down the generations to a youngster that appeared to be in his early teens. I would ask him a question and it would be passed up the generations to be answered by great grandpa and then passed back down through the

generations to me. History questions like age, when was he last dewormed, how long since he has eaten, has he been down rolling, etc. Passed up and down the line. Finally the question about feces. Puzzled look from the youngster. No answer. Has he had a bowel movement? No answer. Has he passed any manure? No answer. Finally stumped for any other phraseology, I said, "Has he taken a shit?" Great grandpa jumps in, "big shit"...Lesson eleven...speak your mind so nobody has to guess what you're saying.

References

Will Rogers, Baxter Black and Shel Silverstein

Non-surgical methods for reproductive management of captive and free-ranging wildlife populations

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Abstract

Fertility control has long been central to reproductive management in captive breeding programs. Species and gender differences in efficacy and safety of contraceptive products have necessitated development and testing of a variety of options, ranging from synthetic progestins to gonadotropin releasing hormone (GnRH) agonists to vaccines against the zona pellucida and GnRH. Specialized centers in the U.S. and Europe provide monitoring, data analyses, and tailored product recommendations to their respective zoo communities. Development and application of contraception or non-surgical sterilization methods for managing wildlife populations has been more challenging. A primary difficulty is gaining access to treat a sufficient number of individuals to achieve population-level impact. Because reversibility is critical in captive populations but often undesirable in free-ranging ones, there is little overlap in contraceptive methods used. Development of better options is needed for both types of programs.

Keywords: Contraception, fertility control, population management, captive breeding

Reproductive management has long been central to successful captive breeding programs. Reversible contraception has been used routinely in zoos since the mid-1970s,[1-3] but efforts continue to identify and develop new methods appropriate for the wide variety of species in managed programs. Interest in fertility control, either reversible contraception or permanent sterilization, for managing free-ranging populations also spans decades but has proven more difficult, primarily due to challenges with effective delivery of contraceptives or application of treatments.

Contraception has been systematically monitored in the U.S. by the Association of Zoos and Aquariums (AZA) Reproductive Management Center (RMC; formerly the AZA Wildlife Contraception Center) since 1990, hosted by the Saint Louis Zoo (www.stlzoo.org/contraception). Annual surveys are submitted to the RMC and compiled in a database that now contains more than 25,000 records of contraceptive use. About 10 years ago a similar program was started in the EU, the European Association of Zoos and Aquariums (EAZA) Group for Zoo Animal Contraception (EGZAC: www.egzac.org). These two organizations work closely, sharing data and a web-based survey data entry system. Availability of some products varies in the U.S. and Europe, but there is considerable overlap as well as shared challenges.

Development and application of fertility control for wildlife has not been as coordinated and focused as for zoos. However, a regularly occurring symposium (Wildlife Fertility Control) with published proceedings serves to bring together scientists and managers with experience and an interest in the topic. The Botstiber Institute, recently established to promote and support fertility control for wildlife management, will henceforth organize and host this symposium.

Although prevention of reproduction is the objective for both captive and free-ranging wildlife, contraceptive reversal to allow genetically important individuals to reproduce is critical in captive breeding programs. In contrast, permanent sterilization is often preferred in free-ranging populations to avoid the need for repeated treatment, highlighting another major difference, which is that captive animals are easier to access or restrain for treatment.

Contraceptives used in captive breeding programs

Progestins

Synthetic progestins were the basis of the first contraceptive methods used systematically in zoo animal populations, beginning with melengestrol acetate (MGA) silastic implants and

medroxyprogesterone acetate (MPA) injections in lions.[1] Because cortisol was more affected by MPA, MGA in implants, in feed or as a liquid to be added to the diet, became the preferred option, although MPA (as Depo-Provera, Pfizer) and megestrol acetate (Ovaban or Ovarid, Schering) are used in special cases. Newer generation implants containing etonorgestrel (e.g., Implanon and Nexplanon: Merck) are available for use in wildlife in Europe but not in the U.S.

The MGA implants (first produced by Dr. U.S. Seal and provided to the U.S. zoo community; now sold by Wildlife Pharmaceuticals, Ft. Collins, CO), have been highly effective in females of all mammalian species treated, once an adequate dose is identified, except equids. However, like domestic horse mares[4] wild equids do respond to altrenogest in the form of Regu-Mate, although the daily oral treatment required is impractical in most cases.

Melengestrol acetate implants are effective for at least two years, and if left in place have been observed to be effective for as long as five years, minimizing handling. Reversibility has not been systematically analyzed in many species (golden-lion tamarins 5, tigers 6), but survey reports from program managers (RMC Database) indicate high rates of reversal in most individuals if the implants are removed.

Early in zoo breeding programs, the taxonomic group most in need of reversible contraception was large felids, e.g., lions and tigers. Unfortunately, felids (and by extrapolation, carnivores) respond differently to sex steroid hormones than most other species. Estrogens and progestins, alone or in combination, stimulate endometrial overgrowth in carnivores if ovulatory cycles are not separated by pregnancy. Studies of progestin-treated felids[7] documented endometrial pathology ranging from hyperplasia to cancer, as well as mammary tumors. Analyses of wild canids revealed similar results.[8] These outcomes have not been observed in other taxa, such as primates and ungulates which now also have a long history of progestin contraceptive treatment, apparently because of differences in the responses of endometrial and mammary tissue to estrogens and progestins.

Combination birth-control pills

Commercially available oral contraceptives made for women are a good option for apes and some Old World monkeys, because of species similarities in response. They have proven safe and effective and are the preferred method for great apes, in particular (RMC Database).[9,10] Although they are safe and effective for primates, they are not an alternative for carnivores. In fact, estrogen priming exacerbates the stimulatory effect of progestins on the canid uterus,[11] an effect that may extend to other carnivores.

GnRH agonists

Due to the risk of pathology from progestin, the GnRH agonist deslorelin (Suprelorin implants, Virbac) has replaced progestins for carnivore contraception (RMC database). In this slow-release form, deslorelin does first stimulate the reproductive axis, but then down-regulation of pituitary GnRH receptors follows. The stimulation phase can be avoided by short-term treatment with oral megestrol acetate around the time of Suprelorin implant insertion, using a protocol developed by Wright and colleagues.[12] An advantage of GnRH agonists is that they can be effective in males of most species as well as females; a notable exception has been male bovids,[13] as has also been shown in domestic bulls.[14] A further advantage is the small size of the implant (grain of rice), which allows insertion by trocar, in contrast to the much larger MGA implants which require a small surgical incision.

Available in two formulations with minimum periods of efficacy of 6 and 12 months, the average period of Suprelorin suppression in the range of species treated in zoos (RMC Database) is more often one and two years, respectively. Lions, however, tend to be suppressed far longer, up to five or even six6 years (RMC Database).

Anti-GnRH Vaccines

Two products sold by Zoetis in the U.S. (Improvest) and elsewhere (Improvac) have been used in zoos, particularly in ungulates (RMC and EGZAC Databases). As with GnRH agonist, they can be used in both males and females. They appear to be safe and effective, although only a small number of

individuals have been treated. Another GnRH vaccine, GonaCon, produced by the USDA National Wildlife Research Center, can only be licensed for use in free-ranging wildlife and cannot be used in zoo animals.

Porcine zona pellucida vaccine

Porcine zona pellucida vaccine (PZP), produced by the Science and Conservation Center (Billings, MT), has been used successfully for several decades in captive animals, primarily in ungulates.[15,16] Advantages include being safe for use in pregnant, lactating and juvenile females, plus natural cycles and sexual behavior continue, which is often preferred. Although being injectable is a further advantage, it requires a booster injection at about one month as well as annually. Reversibility decreases with long-term treatment.

Suppression of reproduction and infertility

Captive breeding programs require reproductive management such that each year some individuals receive breeding recommendations and others do not. Options for those not recommended to breed are separation of sexes or contraception, both of which carry some risk to fertility, again, apparently more serious for carnivores than for other taxa. Synthetic progestins as well as natural progesterone in cycling females can cause endometrial hyperplasia (EH) or other abnormal tissue changes.[17] Even the GnRH agonist implant Suprelorin was associated with increased EH in wild canids unless the stimulation phase, which can induce estrus followed by a prolonged luteal phase.[18] Lifetime Reproductive Planning is being developed by the RMC to optimize breeding intervals that establish and maintain female fertility.

Fertility control in free-ranging wildlife

Although many fertility control methods have been used in many species, few have advanced to management application. Estrogens and progestins are seldom used in free-ranging wildlife, because of potential side effects (especially in carnivores) and because they pose a danger to humans if they enter the food chain (e.g., white-tailed deer). Instead, PZP (porcine zona pellucida) and GonaCon (anti-GnRH) vaccines have been considered more viable options. Major drawbacks have been application of the vaccine, since capture is required, and the need for boosters to establish and maintain contraceptive effect. However, progestins pose little risk for primates; etonorgestrel implants (Implanon: Merck) are being used successfully to control numbers of Barbary macaques.[19]

White-tailed deer have been treated successfully with PZP vaccine[15] in many locations, and more recently GonaCon has been licensed for use in white-tailed deer.[20] Both products have also been used in African elephants for population management and in American bison to control the spread of brucellosis.[15,21] GonaCon is being used for wild boar as well.[22] Although free-ranging domestic horses are not technically wildlife, they have been a popular target species for fertility control.[23] Horses have been treated most extensively with PZP vaccines, particularly ZonaStat-H[15] but also with SpayVac.[24]

Most fertility control products have been used to control mammal populations, and options for other taxa are quite limited. An exception are products containing nicarbazin for pigeons and Canada geese, OvoControl-P and -G, respectively (Innolytics). Nicarbazin, originally developed to control coccidiosis in chickens, has a side effect of preventing fertilization.[20]

Future directions

Although a considerable number of options are available to the zoo community for captive wildlife, there are no reliably reversible methods for male ungulates. Suprelorin, especially with Ovaban treatment around the time of implant insertion, has been safe and effective in all mammalian females tested, but time to reversal is unpredictable, presenting difficulty for planned breeding. As the importance of reproductive management increases for birds and reptiles, more methods, better suited to these species are needed. The challenges for field application are even more difficult, with targeted delivery and longer duration of efficacy being the most pressing problems. Effective population management and

conservation of many species will depend on reliable methods of fertility control. Development of those methods will require research effort and financial support.

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Denver Zoo Mongolia conservation project: effects of progress upon endangered species

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"We did not inherit this wonderful world teeming with life, we borrowed it from our children."
Commonly quoted conservation sentiment

"Humans may be the smartest objects that ever came down the pike of life's history on earth, but we remain outstandingly inept...particularly when our emotional arrogance joins forces with our intellectual ignorance."
Stephen Jay Gould

"The ultimate irony of organic evolution: that in the instant of achieving self-understanding in the mind of man, life has doomed its most beautiful creations..."

"The challenge of this new century is going to be how do we continue to raise the standard of living without destroying the planet..."
E.O. Wilson

Mongolia, "Land of the Blue Sky", is one of the last places left on the planet where people live much as they did hundreds of years ago. It is a wide-open, sprawling landscape roughly the size of Western Europe with just three million inhabitants. Reliable sources estimate there are 50 million livestock in the country. Almost 45% of its people live in the capital Uluanbaatar while nearly 40% are still nomadic pastoralists following their herds. Mongolia is totally landlocked with Russia to the north and China on its west, south, and eastern borders. It sits in the very heart of continental Asia, far from any ocean or sea, nestled in the shadow of high mountains. From the snowy mountains on its northern border with Russia the country rolls down into the high-plateau grassy steppes, which then fade into the harsh, sandy Gobi in the south. With no insulating warm oceans it is an intensely cold place. The growing period for plants is short (late May until September). The average temperature of Uluanbaatar is below freezing, the only world capital to claim that distinction. In the winter of 2010, over 10 million livestock perished in the cold. Periods of such bitter cold, snow, or freezing are called "zuds" where livestock cannot graze and sheep, goats, horses, cattle, and camels die by the thousands. Despite its long, cold winters, Mongolia gets 250 days of sunshine each year.

Under Soviet control from 1921 to 1990, the Russians put an end to over 200 years of Chinese rule. Mongolia was the second communist nation in the world (1921) and the country was virtually closed to Westerners until 1990. After the departure of the Russians, Mongolia has been a democracy since 1991. "Outer" Mongolia is the modern nation while "Inner" Mongolia is the part inside China. A country tremendously rich in natural resources, development of these assets has driven Mongolia headlong into the twentyfirst century and the one time sleepy country is modernizing at an astonishing rate.

In 1996, the Denver Zoo helped obtain roughly 180,000 acres in remote Dornogobi province and established it as one of the country's first Protected Nature Reserves. The area is a semi-arid steppe supporting a unique community of wildlife. Due to the fortunate fact that the area has not been inhabited prior to its acquisition as a Nature Reserve, it retained its relatively pristine ecology. An amazing group of wildlife is native to the park, many of them endangered.

The park is named Ikh Nart (sunny place in Mongolian) and is home to Argali sheep (*Ovis ammon*) the world's largest bighorn sheep; majestic Siberian Ibex mountain goats (*Capra ibex siberica*); Mongolian gazelles and Goitered gazelles. Two wolf packs also live in the park. Rich in bird life, nesting raptors in Ikh Nart include Saker falcons, common and lesser kestrels, black kites, golden and steppe eagles, eagle owls and little owls. Of particular interest are the large nesting aggregations of the cinereous vulture also called the Eurasian black vulture.

The park is unique due to the interplay between the breathtaking native wildlife and the nomadic herders that also live there. In 2001, a collaboration between the Mongolian Academy of Science and the Denver Zoo Foundation initiated a long-term research project at Ikh Nart. The purpose of this project was

aimed to help understand the pressing conservation issues of the area and it has grown into a massive interdisciplinary undertaking involving scientists from Mongolia, from the U.S., from the U.K., from France, and from Russia. In addition, many Mongolian students receive training there in wildlife biology, in veterinary wildlife medicine, ecology, botany, animal behavior, evolutionary biology, and conservation.

On account of this work with students, Ikh Nart was named a model park both in 2015 and 2016 by the United Nations. Supported by the Zoo in their research nearly two dozen Mongolian students have received advanced degrees and go on to make a difference by giving back through teaching future generations of wildlife biologists and veterinarians.

The Denver Zoo is much more than animals in cages. More children visit the Denver Zoo each year than adults go to the Rockies, Avalanche, Nuggets and Broncos combined. For many inner city and underprivileged children, this exposure and connection with some of Earth's most amazing wildlife is transforming and priceless. Also, the Denver Zoo's Conservation Biology Department is much more than window dressing and is deeply involved in projects not only in Mongolia, but also Botswana (wild dogs and vultures), Peru (endangered Lake Titicaca frogs), Vietnam (snub-nosed monkey research), and Rio Mora, New Mexico (bison and land reclamation projects). In each project, local people are included in the research to help insure each area is preserved and protected and its wildlife passed on to future generations.

This discussion will focus on my research with four Mongolian species:

1) Argali bighorn sheep (*Ovis ammon*)
- Their status in the park
- Radiotelemetry studies
- Their disease status and toxicology
- Their natural biology and migrating routes
- Reproductive status
- Geriatrics

2) Siberian ibex mountain goats
- Their status in the park
- Radiotelemetry studies
- Disease status and toxicology
- Natural biology, reproductive status and geriatrics

3) Cinereous vulture (Eurasian black vulture)
- Radiotelemetry studies
- Status within the park
- Natural history and migratory routes
- Disease status and toxicology
- Geriatrics

4) Central Eurasian viper (*Gloydius halys*)
- Most commonly encountered snake
- Aggressive, poisonous snake
- Large numbers are found in hibernacula

In addition, studies with radiotranmitters have been done at Ikh Nart involving wolves, hedgehogs, toad-head iguanas, bats, and the non-venomous coluber constricting snakes.v Mongolian students assist with the studies and data collection.v In this way the Denver Zoo has helped train a whole next generation of Mongolian biologists, veterinarians, and wildlife researchers.v This commitment of the Denver Zoo at Ikh Nart is valuable to the people of Mongolia, but vital to the conservation and preservation of the endangered species at Ikh Nart Nature Reserve.

This presentation will be richly illustrated and supplemented by videos of my research in Mongolia and our work with the endangered species. Videos of the vulture work, argali capture, snake

research and Mongolian students will be displayed. In addition, a compilation of shorts capturing Mongolian daily life will be included.

For the existence of Zoos to continue to be justified, the roll of zoos in the conservation and preservation of both endangered species and their habitat must be continued, expanded, and perpetually committed. The zoos have an obligation and a responsibility to be involved with the conservation of the animals that they display.

These animals on display must become ambassadors for their species, educating us as to their plight and imploring and insisting for their protection and conservation. I hope that you like these videos.

Using amphibians as sentinels for environmental toxins: if we save the frogs, maybe we can save ourselves

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"There are 10 million other forms of life on this planet. We were given this wonderful biodiversity and out intellect. All the other creatures are waiting for us to use that intellect to save our world."
"In conservation work, our successes are temporary and our failures are permanent."
"When a species goes extinct, the world becomes a less interesting place. Currently, we are in a sixth wave of mass extinctions, 100 times the rate seen at the end of the dinosaurs. This extinction rate may go to 1,000 times that rate in the next thirty to fifty years."
"Each species, no matter how inconspicuous and humble, is a masterpiece of biology in itself and deserves protection from the onslaught of humanity."
E.O. Wilson

"The frogs are singing, the frogs are singing! Summer has surely come."
William Shakespeare

Amphibians (frogs, toads, salamanders, newts, and caecilians [the limbless amphibians]), on account of their life cycle, are uniquely sensitive to environmental pressures. By the late 1980's it became apparent that amphibians worldwide were in decline. In areas where they previously were abundant, the golden toads of Costa Rica, national parks in the United States, salamander study sites in Mexico, and in the English breeding ponds, amphibians were demonstrably shown to be in decline or to have disappeared altogether.

In general, the public associates amphibian-like history with biphasic (amphi – "both", bio – "life") life cycle in which adults move to a body of water to breed and deposit eggs that then hatch into tadpoles or larvae. After a varying amount of time (days in spade foot toads, years in pond frogs), the larvae metamorphose into juveniles. Juveniles then disperse to other semiaquatic (pond side, streamside) or terrestrial habitats (with sufficient humidity and moisture) and become adults. Many move hundreds or even thousands of meters from the original aquatic breeding site.

Thus when their lifestyle is more critically examined it is much more complex than simply a life in the water and a life on the land. Each stage of their life cycle can have several compounds. The type of body of water, seasonal temporary pond, permanent pond or lake, running water – gently running stream or moving river, depth of the water of habitat preference for region in the water column, will naturally dictate the life cycle of a species. Habitats are not uniform, and certain types of habitats are used at certain times of year by different life stages of amphibians. Water temperatures can vary tremendously, as can water depth, amounts of water, and abundance of specialized food sources (insect larvae, etc.). Corridors must be kept available between breeding ponds and terrestrial habitats. These habitats are used at different times of the year by different life history stages and species are exposed to vastly different sets of physical, chemical, and biotic conditions within their various habitats. The complexity of the overall habitat requirements and the various nature of their life history makes amphibians especially vulnerable to perturbations in their environment. Amphibians are particularly vulnerable to even slight modifications in their environment. As wetlands, marshes, and pond bottoms become even scarcer, amphibians suffer the change. When waters recede or vanish, they have nowhere else to go.

Amphibians are particularly sensitive to habitat destruction and equally as significant a threat is habitat fragmentation. Habitat destruction is considered the most serious pressure regarding amphibian species. As human populations increase at their present astonishing rate, major land use practices impacting amphibians include agriculture, silviculture, industry, and urban development. These practices typically include filling or draining of wetlands that serve as amphibian breeding grounds, removal of

trees or vegetation used for feeding and as refuges by adults, or alteration of the hydrodynamics of stream and river ecosystems regarding amount of water available.

Even small wetlands house a rich biodiversity. Habitat destruction may be far more difficult to recognize than it might initially appear. Aquatic environments are the ultimate sink for most chemical toxins regardless of their source (agriculture, industry, housing developments). These chemicals applied to forests, lawns, golf courses, agricultural fields, and city streets can ultimately and directly expose larvae, juvenile, and adult amphibians to harmful levels of herbicides, insecticides, fertilizers, ice melts, and industrial chemicals. The problem is ponds, lakes, streams, rivers, or fields heavily contaminated by industrial chemicals or pesticides may superficially appear fine, even though original species composition, ecosystem function, and original inhabitants have already been completely eliminated and destroyed.

Habitat fragmentation is more than loss of ponds, trees and streams. Habitat fragmentation may restrict movement of amphibians within their home range and reduce the potential for reproduction and recolonization. As populations become isolated and the number of individuals per population is reduced, the potential for inbreeding is increased, demographic structure may change, less successful reproduction may occur, and the number of birth defects and abnormalities may increase. Likewise, the amount of pollutants, pesticides, and harmful chemicals can lead to the ultimate collapse of an aquatic ecosystem and an increased number of genetic malformations.

Now let us focus upon some findings concerning amphibian status within our own state. Once common in the Southern Rocky Mountains, the boreal toad *Bufo boreas* has experienced dramatic declines in population over the last 25 years. Surveys completed in the 1990's indicated the toads were present in only one of 377 sites historically plentiful in western Colorado and could be found in only 17% of known reported sites along the Colorado Front Range. Once extremely common in Rocky Mountain Nation Park, boreal toads are now only found in seven localities with just two of these populations likely to be successful each year. Only 50 active breeding sites have been found in the entire state. Of these populations, fewer than five egg clutches were documented as laid per year, these populations may not survive over the long term.

The steady decline in the Earth's biodiversity is an unintentional consequence of multiple factors enhanced by human activity. The factors can be summarized by the acronym "HIPPO" with the first letter corresponding to the cause and rank of the destructive force.

H – Habitat loss (including that caused by climate change)
I – Invasive species (alien predators, new disease organisms, dominant more efficient competitors)
P – Pollution (all types)
P – Human overpopulation (the root cause of the other four factors)
O – Overharvesting (overfishing, overhunting, overgathering)

It is tempting to look to one "smoking gun" when it comes to a species in decline but usually two or more factors are at work. When a population is reduced to a small area due to habitat destruction, it is more susceptible to pollution, predation, overharvesting, disease, and inbreeding. Our work as conservation biologists is to identify and single out the impact of the most destructive pressure and then to initiate actions to nullify the pressure. A pressure caused by the action of human beings is called an "anthropogenic" factor. American wetlands and waterways bear sad testimony to these human based pressures. Decline in wetlands has contributed tremendously in the decline of amphibians. In addition, natural phenomena in concert with anthropogenic effects can also be taking a toll. Examples of pressures at work on one such Colorado amphibian species include:

1) Destruction of habitat.
2) Fragmentation of habitat.

3) Biocides (herbicides – both terrestrial and aquatic fungicides, insecticides, rodenticides and agricultural fertilizers).
4) By-products of mining and industrial pollutants (coal, lead, heavy metals, PCB's, plastics) water pollution by automotive and railway products.
5) Predation on larval and metamorphosing stages – fish, birds, turtles, snakes, raccoons, foxes, feral cats, bullfrogs, predation on tadpoles – fish, snakes, birds, insects, predation on eggs – fish, birds, leeches, bullfrog tadpoles, juvenile snakes.
6) Acid rain, high nitrate accumulation, low dissolved oxygen content of water, human wastewater and sewage, and fecal contamination of water by cattle, hogs, horses, goats, or Canada geese.
7) Ultraviolet light (UV-B).
8) Chytrid fungus infection of the skin.
9) Parasitism, ingestion of blue-green algae by tadpoles, competition with other frog species, disease, morphological abnormalities by pollution of inbreeding.
10) Prolonged drought, unseasonable temperature extremes – too early high temperatures can lead frogs and toads to breed too early and offspring do not survive later snow and cold spells. Flooding. Overgrowth of vegetation which eliminates open water and created too much shade.
11) Siltation. Erosion and collapse of shores, degradation of breeding areas by fish, trampling of good shoreline by cattle.
12) Highway construction, strip mining, construction of golf courses, using marsh as landfills, draining and filling marshes for development.
13) Agricultural cultivation and associated farming practices.
14) Recreational development of aquatic sites for swimming, boats, and introduction of fish, mowing around ponds, burning of vegetation around ponds and filling in tributary ditches.
15) Destruction of ponds and marshes during development of residential subdivisions and subsequent urbanization.
16) Contamination by road de-icers and automotive wastes, lining irrigation ditches, canals, ponds, and marsh areas with concrete.
17) Ignorance about a species and widespread indifference about its plight among county, state and federal officials. As is true for many declining amphibians and for many declining species – this may be the most serious threat.

Most amphibians are influenced by a number of the threats mentioned. Also, the threats may vary in time and have different impact and significance at different sites.

In addition to boreal toads, our discussion will examine the effects of substances in the environment such as estrogen, antibiotics, and thyroid hormone and what effects these molecules may have on local salamander species (*Ambysoma tigrinum*). Finally, although amphibians have been the primary focus of our discussion, we will investigate reptiles acting as a reservoir for such diseases as West Nile Virus and their role in transmitting *Salmonella* infection to human beings.

Discussions like this are important since they demonstrate the "one-health" philosophy of world well-being and the veterinarian's role in monitoring the health of our environment. The condition of local amphibians tells much about the overall health of an ecosystem.

People ask me, "Why do you study and save frogs?" I say, "Maybe by studying and saving frogs we can learn how to save ourselves." Veterinarians are the stewards of more animal wellness than merely dogs and cats. We must stay involved. There is enough time to make a difference but we must act now.

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Diseases caused by canine herpes virus

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Canine herpes virus (Canid herpes virus, CaHV-1) is a member of Family Herpesviridae, subfamily Alphaherpesvirinae. Canine herpes virus has world-wide distribution. In Scandinavia, about 86% seropositivity to CaHV-1 has been reported in unvaccinated bitches. Canine herpes virus serological incidence varies in different countries of the world (40-93%). Canine herpes virus host range is canids (dogs, wolves and coyote) due to receptor specificity of the virus. Canine herpes virus does not infect humans. Similarly, human herpesviruses do not infect dogs. Canine herpes virus is monotypic and CaHV-1 is genetically closely related to feline herpes virus. Because CaHV-1 is poorly immunogenic, the diagnosis can be missed based on serology alone. Recovery from herpesviruses is associated with lifelong latent infection with localization to nerve ganglia. Latently infected dogs can convert to seronegative status.

The virus is transmitted by various routes such as oral, aerosol transmission through respiratory route, coughing, sneezing, and *in utero* route leading to high puppy mortality short time after birth. Canine herpes virus is very labile in the environment. Thus, successful transmission of the virus requires direct contact with nasal and genital secretions. The bitches remain asymptomatic and puppies become anorectic.

Canine herpes virus produces latency in trigeminal and lumbosacral ganglia. Abortion and still birth puppies may also be associated with CaHV-1 reactivation. No CaHV-1 reactivation occurs from PBL. Canine herpes virus is latent in dogs, worldwide. Virus shedding occurs sporadically when animals are stressed by high population density, transportation, pregnancy and immunosuppressive therapy (glucocorticoids). The predisposing factors in newborn puppies include exposure to cold weather leading to hypothermia that allows higher replication of the virus. Keeping the puppies warm is important.

Clinical signs in neonatal puppies include vocalization, anorexia, loss of sucking reflex, abdominal pain, yellow-green soft feces, dyspnea and hypothermia. Petechial hemorrhages occcur on mucus membranes. Incubation period is about seven to ten days. Litter mortality of about 100% is possible. Older puppies remain asymptomatic and mount a febrile response. Animals that survive systemic infection can develop persistent neurologic signs, such as ataxia, blindness and cerebellar vestibular deficits.

Reproductive disorders include lesions on penis in male dogs and vaginal hyperemia and lymph follicular lesions in females. In pregnant females, CaHV-1 causes mid-gestation abortions and stillborn puppies.

Respiratory diseases due to CaHV-1 include mild rhinitis, pharyngitis, and pneumonia. Canine herpes virus infections are detected about three to four weeks after introduction of dogs in a kennel. Underlying factors such as immunosuppressant drugs during cancer therapy can exacerbate CaHV-1 induced tracheobronchitis in mature dogs. I have diagnosed few cases of pneumonia in adult dogs with widespread infection of lungs confirmed by CaHV-1 fluorescent antibody test. There are recent reports of CaHV-1 associated acute respiratory disease in three breeds of dogs. The lesions included hemorrhagic rhinitis and tracheaitis. No other CIRDC pathogens were detected in this case report of CaHV-1 associated lung disease.[1]

Ocular disease due to CaHV-1 has been extensively studied, recently. The disease symptoms include conjunctivitis, blepharitis, keratitis, and recurrent eye infections. In newborn puppies, eye infections are bilateral associated with blindness, cataracts, optic nerve atrophy, and retinal degeneration. Dendritic conjunctival ulceration is strongly suggestive of CaHV-1 infection of eye. Spontaneous reactivations of CaHV-1 associated ocular infections are not common.

In adult dogs clinical cases of systemic CaHV-1 have been reported. Lesions include multifocal hepatic necrosis and intranuclear inclusions in liver, adrenal and small intestines. The role of CaHV-1 in neurologic disease needs more work. In other canids, such as Iberian wolves, CaHV-1 infections have been documented.

Canine herpes virus lesions include multifocal areas of necrosis and hemorrhage in lungs, liver, brain, and kidneys. Lesions in very young puppies include red hemorrhagic spots on kidneys. Canine herpes virus causes necrotizing vasculitis. Lymph nodes are swollen and the spleen is enlarged. Genital lesions in older females include vaginal hypraemia and submucosal hemaorrhages.

Specimens of choice include fresh frozen kidneys, lungs and liver. For kennel diagnosis, CaHV-1-SN is a test of choice. Canine herpes virus is transmitted to newborn puppies in the birth canal. A breeding kennel can check the potential shedding of CaHV-1 in vaginal swabs just before whelping.[2] If the virus is detected, cesarian section can be used to deliver the puppies to prevent transmission of the virus.

The role of CaHV-1 in canine diseases is expanding because of the availability of more sensitive tools such as CaHV-1 PCR on formalin-fixed tissue sections. If a kennel is experiencing fertility problems, testing for canine herpesvirus exposure by SN test and canine Brucella card test are recommended. Serology indicates that dog has been exposed but may or may not be shedding the virus.

Once the symptoms develop, treatment of puppies with signs of systemic disease has a poor prognosis. Antiviral therapy for CaHV-1 ocular disease includes cidofovir ocular drops 0.5%, one drop twice daily. Trifluridine ophthalmic solution (1%) has been found to reduce virus shedding and lesion scores.[3] dministration of immune plasma by the intra-abdominal route (one to two ml) has been reported to provide some protection.[4] Immune sera can be prepared by pooling sera of bitches that have given birth to CaHV-1 infected puppies. Rearing the puppies in increased temperature (98-100°F) and 50% relative humidity in an incubator reduces losses. Under experimental conditions, the raising the environmental temperature has reduced puppy mortality and lesions. After antiviral therapy, puppies still may have residual lesions in brain and myocardium.

Introduction of CaHV-1 in a kennel can be prevented by serological testing all newly introduced animals for breeding purposes. Problems have been reported in breeding kennels with introduction of an infected animal that can potentially shed CaHV-1 for one week. However, if the CaHV-1 is accidently introduced into a breeding kennel eradication is difficult. In a breeding female, subsequent litters from an affected bitch have a low risk of developing clinical CaHV-1 illness.

A vaccine for CaHV-1 is available in Europe (Eurican herpes 205; contains enriched glycoproteins of the CaHV-1) but not in the USA. A titer of 1:4 on CaHV-1-SN is protective. Vaccination is performed towards the end of pregnancy and boosted at one to two weeks before whelping. The vaccine has been reported to be effective and safe in pregnant bitches. Although the vaccine CaHV-1 is attenuated, vaccine virus can establish latent infections. Due to the low frequency of CaHV-1 outbreaks, demand for commercial CaHV-1vaccine has been less. Canine herpes virus does not survive well outside the host and is susceptible to most disinfectants used for cleaning the surfaces.

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IVF in the dog: recent advancements and future directions

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Introduction/abstract

Development of assisted reproductive technologies (ART) in the domestic dog, including *in vitro* fertilization, has lagged behind other species. This can be attributed to some unique challenges in dogs, including prolonged anestrus periods resulting in infrequent ovulation events,[1] the 2-3 day post-ovulation delay in oocyte maturation,[2] and the all-encompassing bursa obscuring access to the ovary and oviduct. Despite these challenges, significant effort has been dedicated to understanding domestic dog reproduction because this species holds great promise as a research model for both human medicine and endangered species of wild canid, as well as its inherent value as a beloved companion animal. This review describes advances in ART in the domestic dog leading up to the successful production of offspring from in vitro fertilization (IVF)-derived, cryopreserved embryos,[3] then provides a future perspective for the improved efficiency and broader applicability of associated technologies in canids, with specific emphasis on *in vitro* oocyte maturation, embryo transfer, and embryo cryopreservation.

The domestic dog as a research model/applications of IVF

The applications of *in vitro* fertilization in the domestic dog are diverse. First, IVF introduces the ability to transfer gametes or embryos to distant locations, rather than the animals themselves, to produce live births. As the closest domestic relative, the dog is the ideal model to develop the technologies to maintain genetic diversity in small populations of rare canids, such as the endangered African painted dog.[4] Second, there is interest in understanding canine reproduction for the propagation of rare or working breeds of dogs. For example, guiding eye dogs are typically neutered prior to the completion of training, thus the most apt individuals are often not represented in the breeding pool. Efforts to develop ART in Labrador retrievers have already made large strides toward this goal.[5] Finally, though most research in the field of human health uses the laboratory mouse, there is a need for more relevant animal models for study.[6-8] Over 400 genetic diseases have been identified in domestic dogs with homologous afflictions in humans.[9] Dogs also intimately share our environment, and thus are exposed to the same environmental factors that may contribute to disease. Yet, to realize the dog's potential as a biomedical research model, the ability to produce specific transgenic animals is required. Transgenic puppies have been produced via somatic cell nuclear transfer,[10-12] and via CRISPR/Cas9.[13] This is very promising, and further improvement of select reproductive technologies might increase rates of successful transgenesis, or eliminate the dependency on zygotes, which currently limits broad utilization.

IVF and beyond

The recent production of beagle puppies by IVF[3] identified several core changes responsible for the success. First, magnesium supplementation had a beneficial effect on sperm motility and function. Second, *in vivo* matured oocytes were collected 6 days after the luteinizing hormone (LH) surge for optimal oocyte quality. Specific timing of collection was accomplished via monitoring of serum progesterone and LH. These changes significantly increased embryo production rates, though development in culture past the 16-cell stage was not observed. All embryos were vitrified at the 4-cell stage and thawed using the commercially available Vit Kit (Irvine Scientific). Finally, transfer of vitrified 4-cell IVF derived embryos to the oviduct, rather than the uterus, culminated in the live births.

These methods represent a strong first step in advancing assisted reproductive technologies in the dog toward the described applications. *In vivo*-matured oocytes were necessary to develop the initial protocol, but moving forward, use of *in vitro*-matured oocytes from anestrus ovaries would remove the necessity of specific timing of oocyte collection, as well as preserve the future reproductive potential of

the donor animal. Additionally, advancements in embryo handling methods represent areas for substantially increasing success rates in terms of live births.

In vitro oocyte maturation

Several excellent reviews exist on dog *in vitro* oocyte maturation (IVM) efforts,[14-16] therefore work prior to these publications will not be covered. Recent research has focused broadly on a few key areas: reduction of reactive oxygen species, mimicry of the *in vivo* peri-ovulatory conditions, and somatic cell co-culture. Lowering oxygen levels to 5% versus 20% had some benefit, primarily for reducing rates of degeneration,[17-19] and retinoic acid[20] has been shown to improve rates of maturation. Mimicry of the ovarian hormonal milieu has had variable results,[21,22] although supplementation with estrus bitch serum to recapitulate the post-ovulatory environment was detrimental to oocytes[17,23] in recent reports. Mimicking the maturation delay observed *in vivo* via a phosphodiesterase 3 inhibitor improved final metaphase II rates. [24] Finally, co-culture typically has proven beneficial, with dog estrus oviductal cells,[25] bovine granulosa cells,[26] or bovine cumulus-oocyte complex conditioned medium[27] all improving rates of MII.

It is also evident across these studies that: 1) larger diameter oocytes are more competent to mature, [28,29] and 2) there are significant differences among individuals, wherein there will sometimes be one individual for which most or all collected oocytes will reach MII, despite the fact that overall rates rarely breach 20%[18,23] This suggests, as others have already noted,[15] that some aspect(s) of oocyte cytoplasmic maturation are not supported by current IVM protocols. When oocytes are collected from the ovarian follicles of some individuals, likely nearing estrus, a higher proportion are already 'primed' for nuclear maturation. Improved understanding of follicle development in the dog could therefore inform the IVM in this species. Furthermore, with a known-successful IVF protocol, we now have an improved functional end-point for the assessment of future *in vitro* oocyte maturation protocols.

Embryo transfer

Success of domestic dog embryo transfer into the uterine horn is highly dependent on embryo stage and synchrony between donor and recipient estrous cycles. Using fresh, *in vivo*-derived embryos, Tsutsui et al reported 51.9% success rates in the transfer of 8-cell to blastocyst stage embryos into the uterine horn.[30] Here, a window of ± 2 days between donor and recipient still resulted in live birth. Our laboratory had similar success (41.7% or 5 births from 12 embryos), with fresh, blastocyst stage embryos (Table).

It has been reported that the success rate is reduced, but still possible, when zygote to 4-cell stage embryos are transferred to the uterine horn.[31] This is despite the fact that, *in vivo*, embryos do not reach the uterus until ~Day 11 after the LH surge (~16-cell stage).[5] In our hands, live births only occurred when the donor and recipient were precisely synchronized and embryos transferred into the physiologically appropriate site. Our early trials with IVF embryos involved transfer of 2-cell to 8-cell stage embryos into the uterine horn of precisely synchronized recipients. Similar to the results of England et al, the transfer of early stage, IVF-derived embryos into this portion of the reproductive tract, yielded only a few implantations sites and no live births.[3] Oviductal transfer is more challenging due to the bursa which surrounds and obstructs access to the canid ovary and oviduct; in addition, the oviductal tissue is much more delicate than that of the uterine horn.[32] However, this is the physiologically appropriate location for embryos < 16-cell stage,[5,33] and oviductal transfer of IVF-derived embryos via laparotomy and cannulation (Figure) yielded live births where uterine transfer had not.[3]]

Currently, *in vitro* embryo development and cryopreservation protocols (See Embryo cryopreservation) are robust up to the 8-cell and morula stage, respectively. Thus, we can capitalize on these successes by improving our ability to transfer earlier stage embryos. Ideally this would involve the less invasive laparoscopic oviductal transfer, which has made huge strides in recent years in both domestic and nondomestic felids.[34] For the dog, Byeong Chun Lee's laboratory utilizes a highly efficient method of *in situ* oocyte flushing from the oviduct,[35,36] and transfers into this organ are increasingly successful (27 births of 35 auto-transplanted, microinjected zygotes).[37] Still, this procedure is dependent on laparotomy due to the ovarian bursa anatomy. Development of new tools and technologies to access

these organs within the bursal fat pad would allow for more physiologically synchronous and less invasive procedures, which in turn will improve birth rates.

Embryo cryopreservation

Abe et al reported the first successful live birth resulting from cryopreserved canine embryos in 2011, with 9.1% (7 births of 77 cryopreserved 4- to 16-cell embryos) using the Cryotop method.[38] The Cryotop method was preferred over slow freezing for canine embryos between the 8-cell and early blastocyst stage (two births of 35 Cryotop embryos vs. zero births of 25 slow frozen embryos).[39] In our laboratory, vitrified 8- and 16-cell embryos resulted in a 16% birth rate (1 out of 6 embryos) using a closed vitrification system (Vit Kit). The same system was utilized for the IVF-derived embryos in our laboratory (2- to 8-cell stage), with results depending on transfer location, as previously described.[3]

The high lipid content in dog embryos has historically hindered the vitrification process,[40] as was the case in the pig.[41] Development of delipidation or lipid polarization protocols prior to vitrification might improve cryosuccess in dog embryos, as it has in swine. This involves centrifugation to polarize lipids, followed by microaspiration for full lipid removal. Delipidation has been critical for the production of live piglets after cryopreservation and transfer of porcine embryos.[42,43] Partial lipid polarization (i.e. reduced versus fully delipidated) resulted in live births with both cryopreserved domestic cat oocytes[44] and embryos.[45] As little is known of the function of the high lipid content in dog oocytes and embryos, partial delipidation represents an interesting option for both improving understanding of the role of lipids in dog embryos, and for developing more successful cryopreservation strategies.

Unlike early stage embryos, domestic dog blastocysts respond poorly to freezing attempts. Abe et al noted that the majority of Cryotop-vitrified blastocysts displayed abnormal morphology and significantly reduced viability after thawing.[38] Slow-frozen dog blastocysts demonstrated high rates of blastocoele re-expansion; however, a significant reduction in viability of cells was still observed,[46] Similarly, our laboratory has seen poor viability of early stage embryos, with no pups born after the transfer of 37 vitrified-thawed blastocysts into the uterine horn (Table). Others have suggested that dog blastocysts are sensitive to handling, evidenced by the high frequency of blastocoele cavity shrinkage in freshly collected embryos.[40] Some have deliberately collapsed this cavity in human blastocyst studies, to reduce opportunity for ice crystal formation during cryopreservation,[47,48] and this strategy did not impede pregnancy or birth rates. This strategy may be useful in dog blastocyst cryopreservation as well.

Conclusion

Significant advances have been made in recent years to ‘catch up’ the development of assisted reproductive technologies in the domestic dog with the progress that has occurred in other species. The first successful live births via IVF represent a major advancement of this ART in dogs and a critically needed tool for the evaluation of oocytes produced by other assisted reproductive technologies, such as *in vitro* oocyte maturation. Moreover, renewed focus on embryo transfer and cryopreservation technologies could provide a means to capitalize on the advancements in IVF and transgenesis in dogs, for application to endangered canid conservation efforts, development of biomedical research models, and propagation of working dog breeds.

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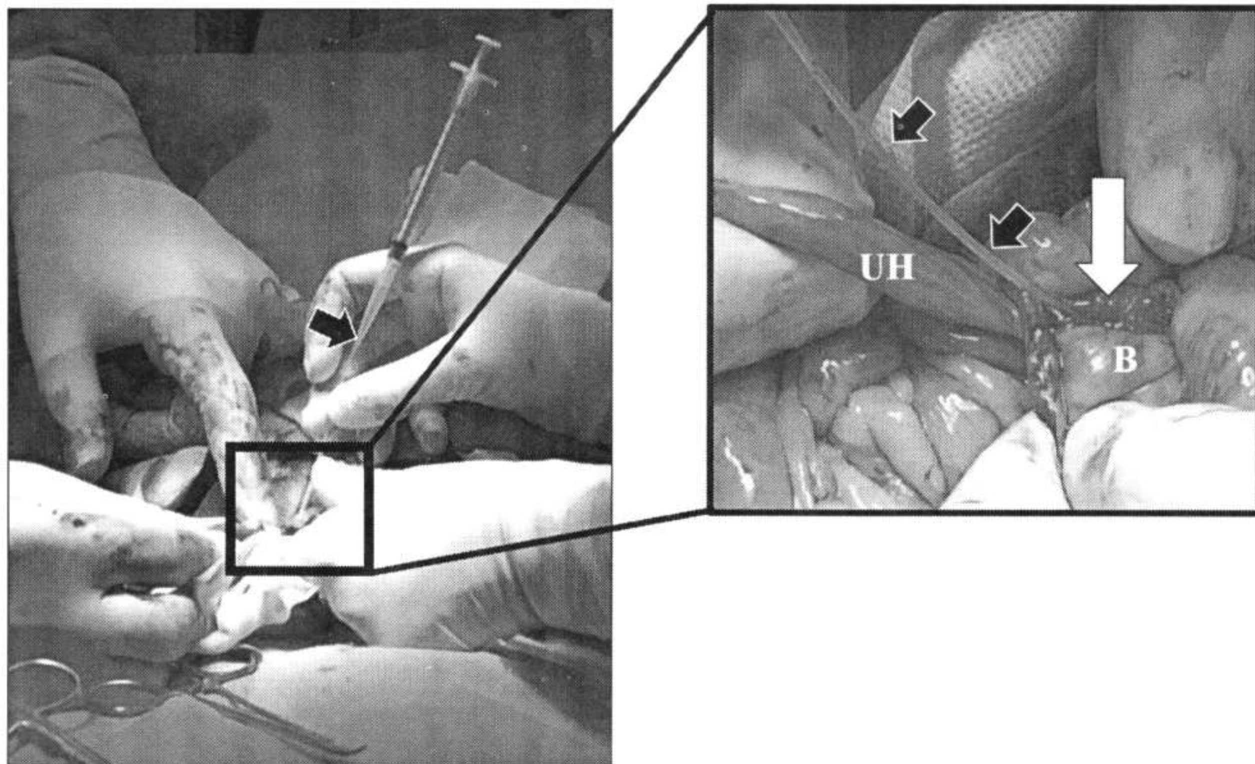
Table: Results of fresh versus vitrified blastocyst transfer in day 14 post-LH surge beagles. Following ovariohysterectomy, embryos were flushed from uteri then recovered in TCM-199 with 15% FBS, penicillin and streptomycin at 39 °C, 5 % CO_2 incubator for maximum 2 hours until transfer to recipient uterine horn via laparotomy. For Vitrification Solution A (VSA), embryos were incubated in base medium (TCM199 + 10% FBS) for 1 min, then in 7.5% ethylene glycol and DMSO in base medium for 3 min. Embryos were transferred in 17% ethylene glycol and DMSO and 0.4 M sucrose in base medium, loaded on an EM-Grid, plunged into liquid nitrogen (LN_2) then stored in a cryovial. For VSB, embryos were pre-incubated in base medium with 7.5 µg/ml Cytochalasin B and 0.1 mM ascorbate for 15 min, then processed as VSA. For thawing, the EM-Grid was held in air for 2 sec at RT and then immersed into 5 ml of 0.5 M sucrose in base medium at 37°C for 1 min. Cryoprotectant was removed by incubating the embryos in 5 ml of 0.25 M, 0.125 M, and 0 M sucrose in the base medium for 5 min successively. The embryos were immediately transferred into recipients.

	Donor ID	Recipient ID	Day of transfer after LH surge	no. embryo transferred	no. of pups
Fresh ET	4199863	3328350	15	2	1
	4477162	3555429	14	4	1
	4511140, 4352661	4335490	**	4	2
	4511573	4513126	14	2	1
	3443141	4452151	17	8	0
Frozen ET	4168224, 4791754	4454464	14	2 (VSA), 2 (VSB)	0
	4611225, 4550099	4652649	14	4 (VSA), 3 (VSB)	0
	4295731, 4550099, 4260597, 4791754, 4295889	4625153	14	10 (VSA), 5 (VSB)	0
	4212916	4331192	14	6 (VSA)	0
	4295889, 4295731	4460162	14	3 (VSA), 2 (VSB)	0

Figure. Images of oviductal embryo transfer via laparotomy for IVF-derived, cryopreserved 4-cell embryos. The left and right ovarian bursas (B) of a recipient hound experiencing a natural estrous cycle were exteriorized, a slit was made through the bursal window to better access the infundibulum (white arrow), and embryos were dispensed via a tom cat catheter (black arrows) advanced approximately 2.5 cm into the oviduct. UH = uterine horn



Look sharp! Acupuncture in canine reproduction

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Abstract

Acupuncture is becoming more routinely used in clinical practice. Current understanding of the neurophysiologic mechanisms of action has increased its use, while Traditional Chinese Medicine has increased in popularity. While scientific papers are lacking for its use in canine reproduction, its use in humans and research in other animals can guide us in this area of practice.

Keywords: Acupuncture, fertility, lactation, pregnancy, parturition

Introduction

Acupuncture is an ancient technique; fish and bone needles may have first been used in China approximately 3000 years ago.[1,2] In 650 BCE Bai-le Zhen Jing, considered an expert in equine acupuncture, wrote the Canon of Veterinary Medicine.[3] Traditional Chinese medicine/veterinary medicine (TCM or TCVM), including acupuncture, was based on thousands of years of observation of patterns of illness and wellness. These concepts are quite different from conventional Western understanding of disease and body function. We now understand the neurophysiologic basis for much of how acupuncture affects the body. Acupuncture's effectiveness is correlated with its effects on the neurologic and hormonal mechanisms of the body. In-depth knowledge of TCVM paradigms is not necessary for the successful use of acupuncture; however it may lead to a deeper or different understanding of complex disease presentations. This author does not consider biomedical acupuncture based on scientific understanding of neuroanatomy-neurophysiology and TCVM acupuncture to be mutually exclusive. We should not ignore the new scientific information that delineates the mechanism of action of acupuncture. We also need not ignore thousands of years of observational information.

Acupuncture typically refers to the use of needles alone (dry needles). Also included in the modality are aquapuncture, the injection of liquid—often B12—into acupoints, and electroacupuncture stimulation (EA). Moxibustion is the use of a burning herb over acupoints or in association with the needles placed in acupoints. The use of acupuncture needles by non-acupuncture trained professionals to provide myofascial trigger point release is commonly referred to as "dry needling" by its practitioners and should be understood as a separate modality from acupuncture.

Physiology of acupuncture

The locations of acupoints in small animals have been transposed from information in humans and other animals (horse, cow), as the use of acupuncture in companion animals is relatively recent. Current research is ongoing to histologically define acupoint locations in the dog. Acupuncture points typically correspond with locations where small nerve bundles penetrate the fascia, are close to nerves, or close to blood vessels which have their own innervation or motor points in muscle.[4,5] The TCM meridians or channels often follow the propagated sensation along a nerve, and some may follow lymphatic channels.[4,5]

A review of specific neuroanatomy and physiology is outside the scope of this presentation, but important to understand when selecting acupoints in treatment. In use of acupuncture for reproductive medicine, knowledge of organ association with specific dermatomes is particularly important. Acupuncture modulates the sympathetic nervous system along with the neuroendocrine system.[6,7] Neuroendocrine effects are achieved via opiate pathways which then influence gonadotropin releasing hormone. While the precise mechanisms by which internal organs and hormonal activation are affected by acupuncture are not as well defined as is acupuncture's effect on pain, the overall concept is that of segmental association. Each segment is comprised of a dermatome, myotome, sclerotome and viscerotome.[8] While these components may move some distance from each other during embryonic development, they retain a shared innervation.[8] Each part of a segment can and does affect the other parts. In general, the sclerotome is the most cranial of the segmental parts, with the dermatome more

caudal and the viscerotome most caudal.[9] Stimulation of the skin or muscle of a particular dermatome or myotome can influence internal organs that have the same segmental innervation via musculo-visceral and cutaneovisceral reflexes.[8] Visceral pathology is projected to the spinal cord via a number of pathways. In humans these include sympathetic fibers to spinal segments C8-L2, via the phrenic nerve to C3-C5, via the pelvic nerves to S2-S4, and via the vagus nerve to the brainstem and C1-C2.[8]

This segmental association reveals one of the difficulties with acupuncture research. Sham acupuncture is often used as a control in these studies, but it is clear that a needle placed anywhere in the body is capable of stimulating the nervous system, by virtue of neurologic connections in that area. If sham needles are placed in the same dermatome as the studied acupoints, there can be a similar effect from the sham needles.

Multiple other physiologic effects of acupuncture have been documented. These include vascular effects and effects on the immune system.[10] With acupoint stimulation there is initial local vasoconstriction that lasts for seconds.[11] This is followed by a prolonged vasodilation phase that may last up to two weeks, increasing perfusion to the area.[12] Acupuncture has been documented to cause enhanced humoral immunity, increased white blood cell count, antibody levels, and phagocytic activity.[12] In some human studies hormonal effects from acupuncture have been documented to last up to three months.

A review of TCVM concepts is outside the scope of this article and is available in other resources.[3,13,14] Identifying general concepts is useful as the terminology is shared in all acupuncture in human and veterinary medicine. Acupoints are arranged on particular channels or meridians, although there are also special points outside these channels. The channels have specific names that include those of particular organs (bladder, gall bladder, heart, etc.). These names refer to the TCVM view of the organ, which is not necessarily, completely, or at all connected to the conventional or Western concept of that organ.[15] When working with acupuncture, it is important to divorce that connection in one's mind and associate the TCVM names with the TCVM meanings as it is truly a different paradigm.

Use of acupuncture in reproduction

Fertility effects-male

Multiple studies in humans have shown acupuncture to increase total functional sperm fraction, percentage of viable spermatozoa, total motile sperm per ejaculate and axonemal integrity.[16-21] Electroacupuncture, but not dry needles in this study, has been shown to increase testicular blood flow.[22] Rat studies have shown positive responses to acupuncture for a variety of androgenic uses. One showed increased total and free testosterone with EA at BL23 and CV 4 for 15 min daily for eight weeks in rats with experimentally suppressed androgen production.[23] Another showed EA at S-29 attenuated oxidative stress and inflammatory response in experimentally torsed rat testes.[24] Still another showed that EA at GV20, CV4, ST36 and SP6 produced enhanced germ cell proliferation and improvement of Sertoli cell function in rats treated with scrotal heat.[25] One canine study presented as a conference abstract showed improvement in spermatozoal morphology, however this study has issues with timing of evaluation, as well as number of patients.[26] As a cautionary note, one study on rats and rabbits at different developmental stages showed that frequent EA during puberty reduced sperm count and negatively influenced gonadotropin releasing hormone (GnRH) level.[27]

Acupuncture, including EA, may be useful in subfertile or infertile adult dogs and considered as an adjunct therapy or primary therapy if no underlying treatable condition can be found for oligospermia or azoospermia of testicular origin. While points may be selected based on a TCVM examination and assessment, empirical points indicated include BL 23, ST 36, SP6, KID 3, KID 7 and BL 20 and 21.[28] Dry needles, aquapuncture or EA can be used. Herbal epimedium powder is often added for testicular issues, including cryptorchidism and infertility.[28]

Fertility effects-female

Definitive work is lacking, but studies suggest that the neuropeptides released due to acupuncture stimulation may act on GnRH.[29,30] If, and how much, acupuncture increases success with IVF in women

is controversial. This is an area where sham acupuncture makes assessment of the studies complicated. Meta-analysis concludes no improvement in conception or live birth when acupuncture is used in IVF, but when each included study is read in total, it is clear that sham acupuncture may be having an effect as well as the chosen acupoints, thus blunting the ability to interpret the results.[31-33] In other studies, lack of an appropriate control group affects study quality.

Some animal studies are available. A rat study showed that acupuncture at BL 36, SP6 and GB9 increased implantation rates in rat blastocysts.[34] One study evaluated repeat-breeder dairy cattle previously treated with GnRH with a 30% success rate.[35] Cows and heifers that did not respond to GnRH were treated with aquapuncture (5-10 ml of 50% glucose solution) at points BaiHui and Shenpeng and "most" animals showed signs of estrus within fourteen days of treatment. After artificial insemination 77.7% had elevated progesterone and 66.6% were palpated pregnant per rectum, however actual delivery occurred in only 44.4%.[35] A study of anestrus sows compared injection of GnRH with acupuncture at points on the back (treatment points) and sham points on the forelegs.[36] Three of four sows treated with acupuncture at treatment points entered estrus, while only one of four did so out of each of the other two groups. In addition, the sows in the treatment group also developed a rise in progesterone.

In bitches with infertility that has been appropriately evaluated with conventional diagnostics without success, as an adjunct to other treatment, and in older bitches with all other appropriate management, acupuncture may be considered. While most cases will be evaluated with TCVM and treatment based on the particular TCVM pattern, there are empirical points that may be used—ones that will address the nerve supply to ovaries and uterus, as well as empirical points based on TCVM uses. These include BL 23, ST 36, SP6, KID 7, Bai Hui, and GV1.[28] Addition of low frequency EA at BL33 and ST 25, CV4 and CV 19 may be of use in older bitches with limited ovarian reserve.[30]

Acupuncture in pregnancy and parturition

Caution is recommended in the routine use of acupuncture during pregnancy[37] Needling the caudal abdomen or back and needling of distal points on the legs is particularly contraindicated, due to the risk of possible miscarriage.[37] This introduces the idea of possible use of acupuncture as an adjunctive treatment in medical abortion and pyometra in the bitch-SP6, LI4, and BL 23-25 may be added to routine medical protocols.

Acupuncture at PC 6 has minimized severe morning sickness (hyperemesis gravidarum) in women in a sham acupoint controlled study.[38] This may be of use in treating bitches that have severe anorexia and vomiting at 3-4 weeks of gestation.

Acupuncture has been used to induce cervical ripening and labor in women.[39] Acupoint BL 67 has been used to accelerate cervical dilation with reported 75% success, although details of the study parameters are lacking.[40] Electroacupuncture stimulation of LI 4 and SP6 resulted in increased frequency and duration of uterine contractions in human and rat studies.[40,41] This author has had success with EA at these and additional points to strengthen uterine contractions during labor in the bitch, equal to or greater than that achieved with oxytocin and calcium administration when observed on a tocodynomometer (unpublished data), although the case numbers are small. The use of EA in this manner is more labor and time intensive than the conventional administration of pharmacologic agents. Use of acupuncture during labor in women reduced the use of meperidine for pain management from 37% to 11%, indicating efficacy in moderating the pain of parturition.[42]

Lactation effects

Acupuncture is reliably effective at inducing lactation. Points used based on effectiveness in human medicine include SI 1 and LI4.[43] Others that can be added include ST 36, SP6, SI 1, BL 17, 20 and 23 or can be chosen based on TCVM examination.[44] The author reports excellent success using SI1 and LI4 alone with dry needles, although EA can be added if needed. Quantitative analysis has demonstrated an increase in the production of prolactin and oxytocin following acupuncture at SI 11 in rats.[45] Other points that have been recommended, depending upon presentation of the patient and TCVM evaluation, include ST 36, SP6, BL 17, 20 and 23.[28,44]

Neonatal resuscitation

The use of acupoint GV 26 for resuscitation and stimulation is widely practiced. Special training and needles are not required for success, as the point, located midline on the philtrum at the junction of its dorsal and medial third, is readily accessed and a 25 g needle can be utilized. Stimulation is provided in a strong manner, with a pecking motion.[46] Stimulation of GV 26 increases blood pressure and stimulates the brain inspiratory centers.[46]

Summary

In summary, while published scientific data on the efficacy of acupuncture in small animal reproduction are lacking, there is both historical information and scientific data in other species that support its use. Study of this modality in companion animal reproduction is needed and encouraged.

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Bugs and drugs: appropriate use of antibiotics in canine reproduction

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Introduction

While the use of antibiotics is necessary and rational in many areas of canine reproduction (pyometra, postpartum metritis, *Brucella canis* treatment, mastitis), their common empirical or unproven use in other canine reproductive situations may cause more harm than good, both to the individual being treated and to human and animal health as a whole. Understanding the health and function of the normal microbiome and the potential adverse effects of unnecessary use of antibiotics can help us form a reasoned approach to their rational use without causing unintended harm. Many questions remain about appropriate antibiotic choice and course of treatment.

Keywords: Reproduction, fertility, normal flora, antibiotic, microbiome

Normal flora in the bitch

Vaginal flora

Multiple authors have documented a range of normal vaginal flora in the bitch, including aerobic, anaerobic, and mycoplasma species. The variety of organisms and differences between studies may be related to sampling procedures, laboratory procedures (including choice of media), regional, management, or breed differences. Organisms isolated from the bitch's vaginal vault include *mycoplasma* spp., *Pasteurella* spp., *Bacteriodes, Streptococcus* spp., *Escherichia coli, Staphylococcus spp., Enteroccus* spp., *Klebsiella* spp., *Proteus* spp., *Bacillus*, *Pseuodmonas* spp., *Arcanobacterium* spp., *Acenitobacter* spp., and *ureaplasma*.[1-7] Few studies looked at anaerobic flora in the vagina, but they were found in approximately equal numbers to aerobic flora when cultured.[7] The types of organisms also vary between cycle stages, potentially due to the medium presented by normal fluid secretions of blood and mucus concordant with cycle stage. A study comparing presence and variety of vaginal flora between owned and stray dogs found no difference in the makeup of the population of organisms.[8] Bacteria originating from skin and the gastrointestinal tract appear to make up the population of vaginal flora. Lactic acid producing bacteria (LAB), specifically Lactobacillus and enterococcus were found in equal numbers in a study of healthy and ill bitches and were not correlated with vaginal pH, age, or body temperature.[9] Spayed bitches with recurrent urinary tract infections had no significant difference in vaginal microbiome than did unaffected spayed bitches.[10]

In most, but not all studies, the highest number of organisms was found during estrus and the lowest in diestrus.[11,12] Increased numbers of organisms may also be found postpartum, but were not associated with neonatal morbidity.[13]

Uterine flora

While some studies documented no flora in the uterus of normal bitches, several others did find low numbers of flora in the uterus of bitches that had normal uterine histology and/or were clinically normal. The largest number of organisms was found in estrus and lowest in diestrus in most, but not all, studies.[11,12] The use of pre-enrichment broth in culturing samples from the uterus was recommended to increase growth from samples from normal bitches.[12] Organisms isolated from the bitch's uterus include *Staphylococcus* spp., *mycoplasma* spp., *Escherichia coli*, *Haemophilus*, *Streptococcus* spp., *Corynebacteria* spp., *Alcaligene faecalis*, *Bacterioides* spp., *Pasteurella* spp., *Clostridium perfringens*, and *Bacillus*.[1,11,14] Culture of postmortem samples routinely grew more organisms and a greater variety of organisms in the uterus than samples obtained from living bitches indicating the microbiome may be rapidly altered postmortem. Preliminary work using extracted DNA and taxonomic evaluation indicated multiple organisms present in the bitch's vagina and uterus.[15] This method of research holds great promise in further delineating the normal microbiome of the canine reproductive tract.

Some organisms appeared more likely to be involved in cases with uterine pathology, particularly *E. coli* although they were also often found in bitches with no pathology.[5,11] E. coli is recognized as the most common organism found in canine pyometra and does recur with successive infections. This may be related to its ability to form biofilms that protect it from treatment.[16] The presence of *Streptococcus* spp. in proestrus was negatively associated with the presence of uterine infections, indicating the possibility of a protective effect.[5] Conversely, in one small study the presence of *Streptococcus canis* or *Streptococcus dysgalactiae* in postpartum vaginal sampling was associated with increased neonatal deaths.[17] In another study, the presence of organisms in the anterior vagina was increased in the early postpartum period but had no apparent adverse effect on neonatal morbidity.[13]

Ovarian bursa

Bacterial growth in the ovarian bursa has also been found, not solely in bitches with pyometra, but also in control bitches (*Enterococcus* sp, *Bacillus* sp. and an unidentified gram positive sp. There are no currently published data on the microbiome status of canine uterine tubes.

Alteration of genital microbiome in bitches

Antibiotics may be used for unrelated infections, for diagnosed reproductive tract infections such as pyometra or postpartum metritis, or given empirically as a preventative or presumptive treatment without clinical indication. Effects on the vaginal and uterine microbiome are likely and the results may allow overgrowth of organisms with increased pathogenic potential by removing competing organisms and allowing resistant organisms to multiply more readily.[19] Treatment of normal bitches with ampicillin or trimethoprim-sulfamethoxazole (TMS) for ten days increased growth of mycoplasma (both) and E. coli (TMS).[19] The use of antibiotics in estrus bitches may adversely affect the attraction of the male dog and his interest in and pursuit of mating.[20] Antibiotic resistance has not been sufficiently evaluated in the bitch's reproductive tract; however one study found a low number of B-lactamase producing and fluoroquinolone resistant organisms in *Enterobacteriaceae* spp. in the urogenital microbiome of dogs.[21]

The use of LAB in positively improving or maintaining a healthy microbiome in the vaginal vault is intriguing, however in one study the oral administration of a probiotic with LAB did not increase the number of bitches from which they were isolated after 14 or 28 days of treatment.[24] Further study of different probiotic compounds, duration of treatment, and a larger treatment group is warranted due to the success of such treatment in other species. Canine vaginal LAB isolates *Enterococcus canintestini* and *Weisella* spp. showed increased bacterial adhesion to canine vaginal epithelial cells as well as inhibition against *E. coli* and P. *vulgaris* (*Weisella*) and *Klebsiella* and *E. faecalis, E. faecium (E. canintestini*). This indicates a potential therapeutic use for canine vaginally sourced LAB in promoting a healthy urogenital microbiome.[23]

Fetal and neonatal concerns

It is not unusual for pregnant and lactating bitches to be treated with antibiotics, either for a documented medical issue that requires treatment (mastitis, metritis) or on an empirical basis by the owner or veterinarian in hopes of preventing a future problem. We do not have canine data evaluating any short or long-term effects of such treatment on the puppies in question, but if they are like humans and rodents then there are reasons to think carefully about antibiotic use without a diagnosed infection. In human infants where the mothers were treated with antibiotics during labor to prevent vertical transmission of group B *Streptococcus* the infants were at greater risk of developing late-onset serious bacterial infections and those infections were more likely to be resistant to ampicillin than infants whose mothers were not treated intrapartum with antibiotics.[24] Several other studies have reported an increase in early-onset antibiotic-resistant neonatal infections in preterm infants when mothers were treated with antibiotics.[25-27] Therefore in humans there can be an increase in both early and late-onset neonatal sepsis, potentially fatal, when the mothers are treated with antibiotics prepartum or intrapartum.

Antibiotic exposure during gestation or parturition can affect development of other morbidities in children. Maternal use of chloramphenicol or penicillin increased the risk of asthma in children by the age of seven years, particularly when used in the first trimester.[28]

Obesity is a serious concern in both the canine and human populations. Prenatal, or early postnatal, exposure to antibiotics increases the risk of obesity in children. In one study children exposed to antibiotics during the second or third trimester had an 84% higher risk of obesity by seven years of age than children who were not so exposed.[29] Interestingly, cesarean section, evaluated independently, also increased risk of childhood obesity by 46%. The connection between perinatal exposure to antibiotics and obesity has also been documented in rodents-this occurred regardless of the later healthy state of the intestinal microbiome in the exposed mouse pups.[30] Not surprisingly, use of antibiotics in the peripartum period also affects the development of the microbiome in infants. Both direct treatment of the infants, as well as treatment of the mother with antibiotics, alter the infant's microbiome, with a lesser effect of the latter.[31] *Bifidobacterium* presence was reduced, enterococci overgrew and continued to increase over the month of monitored time in the study.[31]

Areas for further study

Additional research to understand the canine reproductive microbiome is clearly needed. Evaluation of what constitutes the normal microbiome in the entirety of the bitch's reproductive tract is primary. Current studies have largely ignored anaerobic flora yet studies that looked for it found it in significant numbers. Speciation of organisms such as Streptococcus spp. and Mycoplasma spp. is important in understanding how differences may impact the pathogenicity of the organism. Studies should be performed in living bitches, as postmortem microbiome changes appear to occur quite rapidly. Careful thought must be given to sampling techniques, media used, cycle stage, breed, population, housing, age, parity, reproductive history, and precise location of the sampling so that reproduction of results is feasible and variables controlled. Terminology must be used with precision as colonization is not synonymous with infection. Research on promising LAB that can be used to help prevent overgrowth and sustained infection with more pathogenic organisms is an important and promising area of work, and potentially an urgent one as antibiotic resistance is increasingly a concern. Effects of gestational and peripartum antibiotics on the microbiome in neonatal dogs is another area of needed research.

Knowing when to treat is a serious concern. Failure to treat when needed has serious consequences; however, we often do not consider the significant consequences of treating when inappropriate. Defining those situations is important and could be a goal for the Society for Theriogenology and American College of Theriogenology similar to the ACVIM Consensus Statement on Therapeutic Antimicrobial Use in Animals and Antimicrobial Resistance or the Antimicrobial Use Guidelines for Treatment of Urinary Tract Disease in Dogs and Cats by the International Society for Companion Animal Infectious Diseases.[32,33]

Conclusion

The reproductive tract of the bitch is richly endowed with a diverse microbiome. Understanding it, respecting it, and supporting it are goals we, as a profession, need to support. When treatment is necessary, we must consider the totality of potential consequences of treatment. This is an area with great research potential for the dog, but in the meantime we should make every effort to use antibiotics only when there is a true need, use the antibiotic least likely to affect unintended targets, and to do so for only the necessary time frame. Understanding what the adverse consequences can be may allow us to least anticipate them and possibly ameliorate them as more data become available.

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26,000 whelpings-the high points and high risk successes!

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Parturition management for small animal reproduction has been documented in breeder and veterinary literature for over 100 years. Until the mid-1990's this management was based on a "best guess"; using interventions from subjective symptoms or unreliable parameters such as temperature change. In 1997 Veterinary Perinatal Specialties introduced the WhelpWise service providing monitoring equipment and twenty four hour support services to gather and analyze objective data on parturition, with the goal of improving fetal and maternal outcomes. Our data base now exceeds over 26,000 whelpings. Presented here is retrospective data, collected from an international perspective. This data has been collected in a "real world" scenario of breeders' homes, veterinary offices and service dog organizations.

Our monitored database has included parity, prior dystocias, diet history, progesterone/luteiniing hormone (LH) timing for breeding, X-ray counts, and specific parturition events. The most scrutinized part of our data analysis is maternal/fetal outcomes (live vs. deceased births) and parturition management; evaluating medication doses and frequency and their tocometric response.

WhelpWise is offered as support service for veterinarians, service dog organizations and breeders. The service is only offered in conjunction with the clients' veterinarian; patient care is guided by specific veterinary orders provided by the veterinarian.[1] The WhelpWise service monitors both uterine contractions, and fetal heart rates using obstetrical equipment specifically designed for remote data collection. Data are collected and transferred to a staffed 24 hour monitoring center for evaluation. Using objective data as the base for interventions, assessments and interventions can now be made on objective data. Clients that utilize the service are interested in bettering maternal and fetal outcomes by being proactive in the management of whelping issues. A significant benefit offered by the service is to be able to safely manage inertia through labor augmentation protocols developed specifically for canines, and designed for safe use in the home setting.

Mortality rates for WhelpWise clients

Maternal mortality rates are not well documented for canine whelpings. One study conducted by Moon et al, showed a 1% mortality rate (9 out of 3,410).[2] For clients on the WhelpWise service we have a maternal mortality rate of .011 (3 out of 26,098 bitches). Two of the bitches died from undiagnosed cardiac issues, the third was an undiagnosed diabetic. While it was frustrating to lose these three bitches, the key for success in all three cases was the identification of massive fetal distress in all three litters. The bitches were immediately referred to emergent veterinary care. Cesarean sections were performed but none of the bitches survived after surgery. Thanks to very timely intervention all the puppies in all three litters survived.

Fetal mortality rates for clients on service average at or just under 4%. This number includes known fetal distress that the owner/veterinarian chose not to intervene with operative management. Mortality rates for service dog organizations averages around 1.5%. Research reports 10-33% fetal mortality rate for those not using service.[3]

Our average cesarean section rate for clients that are expecting to free-whelp is 16% (elective cesarean sections are excluded from these data). Reasons for operative intervention when a normal whelping is planned show that cesarean sections are done for abnormal uterine contraction patterns about six percent of the time. These abnormal uterine contraction patterns are frequently caused by a malpositioned fetus, or uterine issues, such as a torsion, an overdistended uterus from polyhydramnios or obstructive dystocia. Ten percent of the cesarean sections were done for fetal distress.

This document will highlight what we have learned with objective measurements during gestation and parturition, and discuss what we have seen to be the most effective management, producing the best outcomes for both normal and high risk pregnancy management.

Temperature change prior to whelping

Historically the most common "way" of "predicting" a whelping was to watch for a "temperature drop" and whelping should occur, depending on the source, in 12-24 hours.[4,5] As described in a prior WhelpWise study, maternal core temperature change occurs in only 33% of bitches, and that change in temperature frequently occurred in an up/down/up/down fashion, not a clear "temperature goes down and stays down" as described in veterinary literature.[4,5] If a detectable temperature change was noted (decrease of > 1 degree Fahrenheit below normal baseline temperature), delivery of the fetuses averaged 37-48 hours after the change rather than 12-24 hours as veterinary resources describe.[6,7]

Length of labor

Labor, as defined for this retrospective review, is the presence of an organized pattern of uterine contractions. Our definition of "labor" is not related to behavioral symptoms of parturition, temperature changes or the presence of vaginal discharge. When using the uterine monitor to confirm the presence of a labor pattern it is not unusual for a bitch to be extremely symptomatic and not be in labor or conversely in very active labor and have minimal/no symptoms and not have experienced a temperature change.

"Labor" is described by a repeatable/predictable pattern of uterine contractions. We have found that labor patterns will vary with breed, litter size, and abdominal mass of the bitch. Uterine contractions are detected by applying an external uterine sensor. This sensor detects changes in intrauterine pressure using a tocodynamometer specifically designed for early gestation human pregnancies. "Contractions" are traced on a linear axis, graphing changes in intrauterine pressure. As the myometrium contracts, the pressure inside the uterus will increase, as the contraction relaxes, the pressure decreases. Uterine activity graphs are documented as time in minutes on the X-axis, and strength of the contraction shown as an increase in pressure in millimeters of mercury on the Y-axis (Figure 1). It is important to note that the bitches are always monitored in a lateral recumbent position. This position will eliminate uterine contractions caused by physical activity. During normal gestation, it is expected that uterine contractions are present in a frequency of one to three per hour beginning about 56 days after the LH surge.

Figure 1. Uterine contraction normal baseline

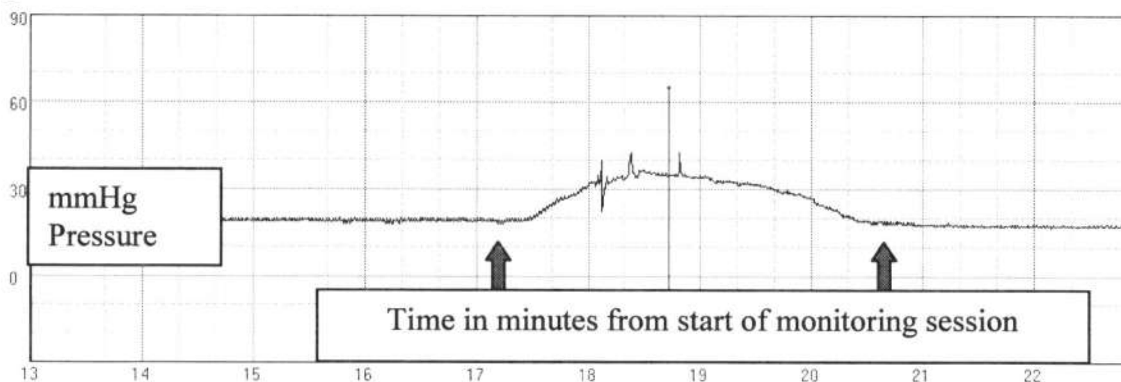
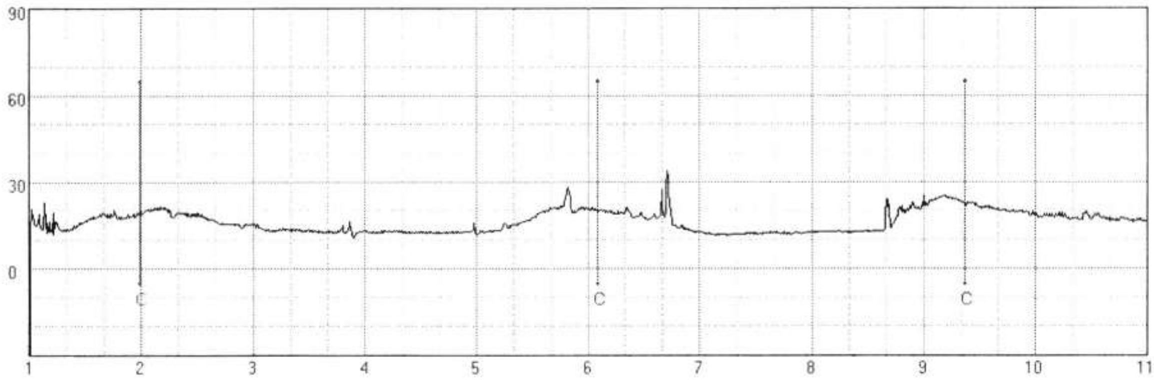


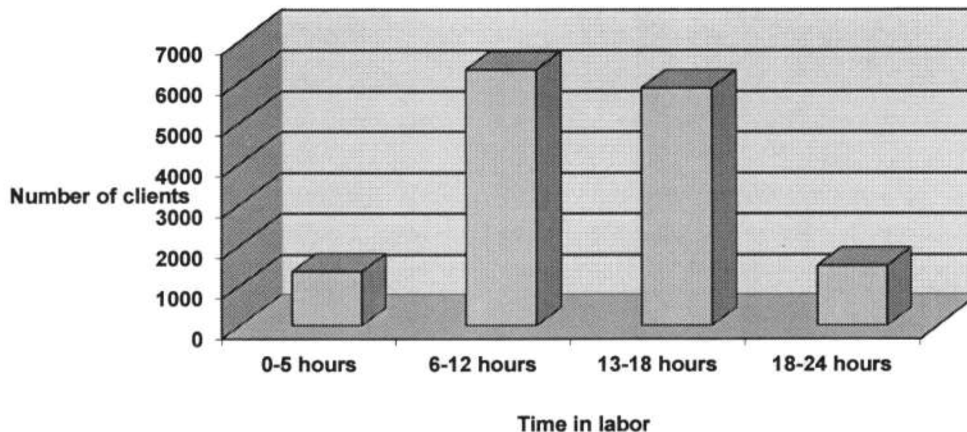
Figure 2. Labor



Presence of an organized pattern of uterine contractions. Contractions are marked with a "C" and red line. Note the consistency in spacing and strength.

Once first stage labor is established, our data strongly support that deliveries will begin on an average of nine hours from the presence of an organized pattern of labor. The range for delivery times averaged eight to 16 hours, with a significant increase in fetal mortality and need for cesarean sections when labor is extended beyond 14-16 hours of an organized labor pattern. This information supports earlier studies, and reflects the benefits of labor management, when first stage labor surpasses the 14-hour mark inertia is usually the issue. The differences were so clear in fetal outcomes related to prolonged labor versus augmented labor, that augmentation protocols were established early in the development of the service. These protocols were based on the human model of parturition management.[8] Human labor management strongly documents that parturition should follow a specific progression once active labor has been established.[8,9] This predictable passing through parturition has been called "Friedman's curve" after the physician who documented that poor fetal outcomes were the result of an ineffective labor; pioneering the concept of human labor management to improve fetal outcomes.[10]

Length of labor before first delivery



Inertia

For the purpose of this retrospective review the term inertia will be used to describe failure to maintain an adequate contraction pattern. Inertia may be the inability to move into second stage labor, or once second stage has occurred, the lack of contractility to continue to deliver pups.

Primary inertia

The incidence of primary uterine inertia has been an extremely rare occurrence (less than .1%). What is demonstrated using the WhelpWise service, is an attempt by the bitch to establish first stage labor. This attempt labor is frequently asymptomatic, and will not be associated with a change in temperature. It is not uncommon for maiden bitches to establish a short episode (three to four hours) of mild contractions that are in a disorganized pattern that will subside and return within 12-24 hours; moving into an active labor pattern. If this attempt to establish labor re-occurs more than twice without progression to active labor we have found that there is a strong correlation with dystocias; either an over distended uterus from polyhydramnios, fetal malposition, or exceptionally large pups being a common cause.

Secondary inertia

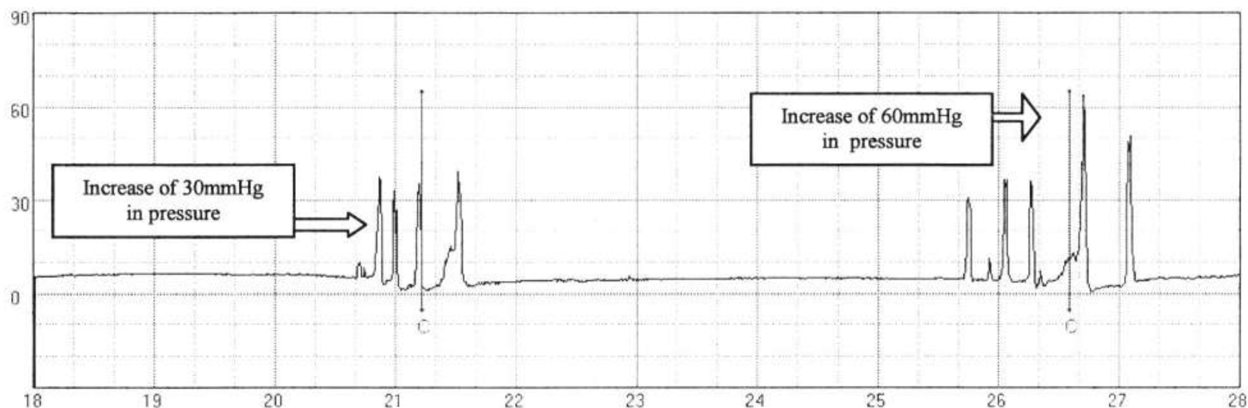
Secondary inertia is considered after the bitch has maintained a contraction pattern for more than seven to eight hours and we begin to see a loss of uterine contraction strength, contraction frequency, or combination of both. Using the human model for medical management of secondary inertia, human fetal outcome data consistently show that lack of progression (cervical effacement, cervical dilation and fetal head engagement) with an active labor pattern requires medical intervention, as low Apgar scores are frequently associated with prolonged labor.[10-12]

Early studies of whelping outcomes associated prolonged active labor with fetal loss. What showed improved fetal and maternal outcomes was early intervention with uterotonics when inertia was detected. Managing the inertia drastically reduced fetal demises from 33% to around 6% in our early monitored clients. Also, noted in the study was an increased incidence of fading puppy syndrome in the unmanaged group (19%) compared to the labor managed group (1%).[7] Secondary inertia, or the inability to progress from either stage one labor to stage two, or maintaining an appropriate interval between deliveries in stage two appears to be the primary cause for fetal mortality and morbidity in our client population.

Whelping management protocols have been modified over the last 17 years of data collection, fine-tuning the "art" of whelping management. When inertia is detected early we expect that 80% of our clients will respond favorably to medical management and proceed with a normal, but augmented whelping. Titrating small doses of either oxytocin, injectable calcium, or both, returning the bitch to her "normal" labor pattern without causing uterine hyper-stimulation or uterine tetany has proven to be an effective and safe way to manage secondary inertia.

Evaluating a bitch in labor (subjectively) is very difficult. As demonstrated with the uterine monitor session below, there is absolutely no inherent contraction strength in the labor pattern, but the veterinarian evaluating the case felt that they observed that the "contractions were strong". What the veterinarian was evaluating was the abdominal expulsive efforts of the bitch, as she was indeed pushing very hard. However, if the contraction strength was increased by labor augmentation, the bitch would require less physical effort to deliver the pups.

Figure 3. Pushing with severe inertia: spikes are caused by increased abdominal pressure as bitch bears down.



Managing inertia

Injectable calcium. The use of injectable calcium to assist parturition has been documented as an uterotonic since 1947.[11] Uterine muscle is dependent on adequate calcium levels to contract effectively, and creates its own calcium consumptive state.[5,12,13] It is this author's opinion that frequently calcium levels can be within a "normal" range based on traditional laboratory values, but the bitch may be experiencing a calcium-based inertia, as her calcium levels may have changed (decreased) within that normal range, causing decreased contractility of the uterus. Inadequate calcium levels are also suspect in bitches that will establish a pattern of labor and then stop contracting. Questioning the ability of the parathyroid gland to rapidly respond to a declining calcium level because of fetal consumption, or active labor we have seen that calcium supplementation both oral and injectable have frequently supplied the needed correction to achieve an effective contraction pattern. We have objectively noted that administration of calcium will increase the strength of the contraction rather than the frequency of the contractions. A study of serum calcium levels conducted at the Guiding Eyes, also has documented that low ionized calcium had direct impact on stillbirths.[13] Additional studies completed by Hollinhead et al, discussed the issues with the availability of free calcium for adequate labor also being impacted by acute inappetence prior to whelping and respiratory alkalosis.[13])

Figure 4. Miniature Schnauzer, labor pattern X 10 hours, beginning of inertia

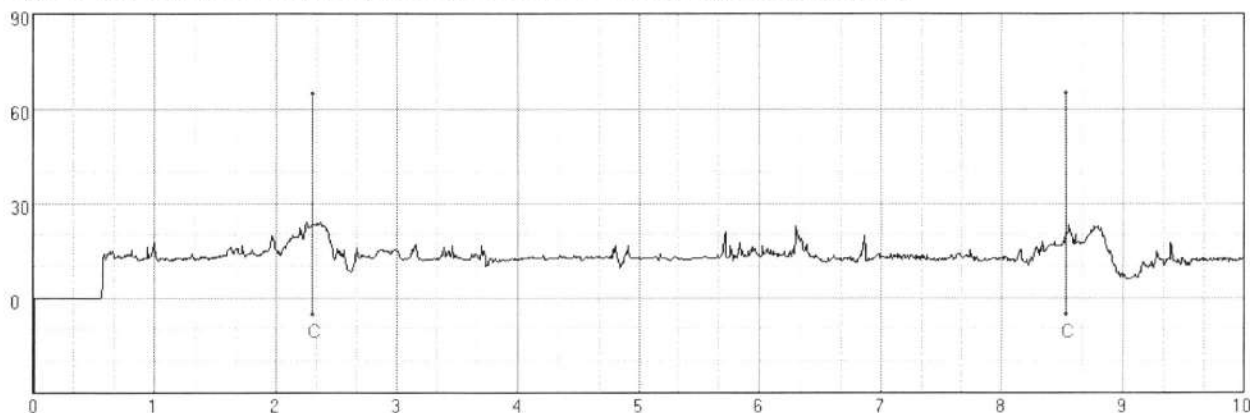
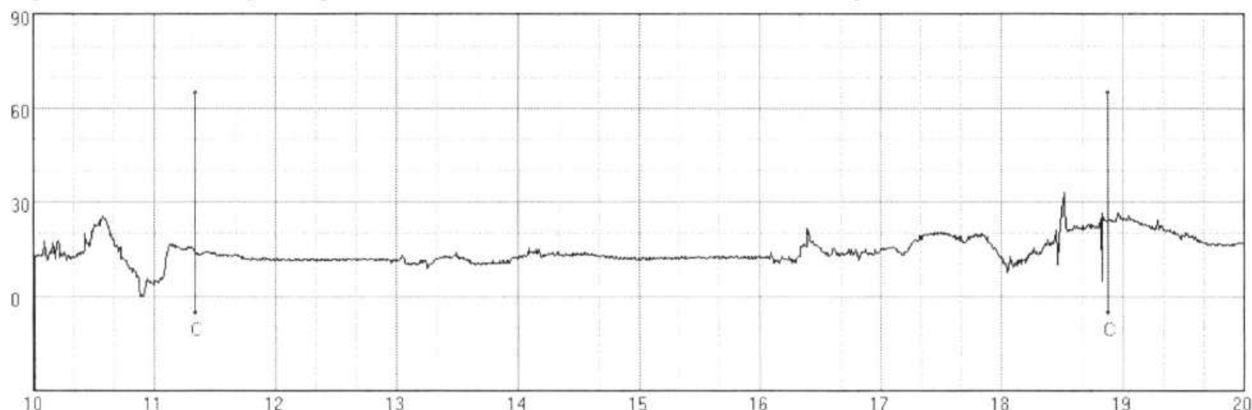


Figure 5. Session beginning 20 minutes after administration of calcium gluconate 10% SQ



Note not only the increase in strength, but also the improvement of overall shape and duration of the contraction.

We have objectively measured the effectiveness of calcium use during labor. Optimally, injectable calcium will provide the best route for administration. Dosage range for calcium gluconate has been successful at ½ to 1 cc of 10% CaGl per 10 pounds of (gravid) maternal body weight with exact doses being titrated to the uterine contraction pattern. Calcium gluconate, administered subcutaneously has been safe. We have experienced no untoward side effects in our patient population unless a stronger concentration of calcium has been used (23%). Injection sites using 23% calcium have been very painful for the animal and a corresponding tissue slough have been reported. No cardiac problems have been experienced with the subcutaneous administration of the 10% solution because of the gradual absorption of the medication. We have had no reported incidence of accidental IV infusion.

In our client population, oral calcium supplements have been successful for proactive prevention of a calcium-based inertia for exceptionally large litters or clients that are not feeding a balanced diet. Both groups appear to be somewhat predisposed to calcium imbalances. Beginning oral supplementation of 500-750mg two to three days prior to parturition or as early first stage labor is established does seem to be beneficial, without noted complications of antepartum eclampsia. Presence of adequate vitamin D levels in the diet has also been key to the prevention of calcium-based inertia. The presence of vitamin D is frequently markedly decreased to completely absent for owners feeding the raw diet, and whelping complications related to this perceived imbalance are frequent.

We have not seen a corresponding improvement in contraction patterns from the oral calcium gel products, and have had several dogs using these products experience gastrintestinal bleeding after their use. Many of these "gel" supplements contain toxic levels of vitamin D when dosed by weight.

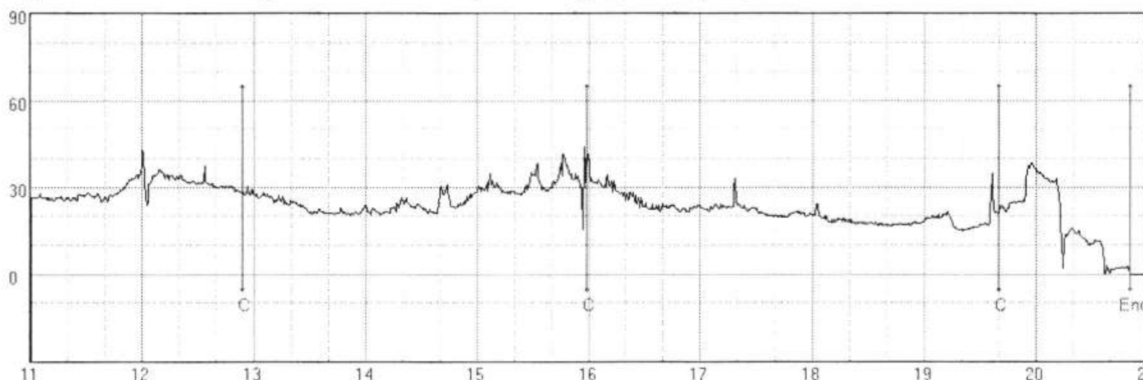
Questions unanswered: Is there a role for "intelligent" calcium supplementation two to three days prior to a whelp, especially with large litters or inappatent bitches? Would this supplementation improve labor patterns, decreasing inertia? Should supplementation be based on litter size/diet of the dam, making sure that adequate levels of vitamin D are also present in the diet?

Oxytocin. Oxytocin has been the most frequently used drug for labor augmentation. Oxytocin dosing prior to the use of the uterine monitor was arbitrary, usually based on animal weight, not uterine contraction patterns.[16] Administering oxytocin in excessive amounts can be detrimental to both labor progression and fetal well-being, as a hyperstimulated uterus does not contract effectively and the constriction of the myometrium will impede blood flow to the fetus. The relaxation phase between each contraction is important to allow blood to circulate to the fetus. Excessive doses of oxytocin can cause uterine rupture. According to our database, effectiveness of oxytocin is related to length of labor, with best response noted after first stage labor has been present for at least eight hours, but not over 16 hours. Administering oxytocin before eight hours of active labor (documented contraction pattern, not symptoms or temperature change) or after 16 hours of labor frequently has minimal effect on the contraction pattern.

From our perspective, oxytocin dosing should always be titrated to the existing uterine contraction pattern, without regard for body weight of the bitch. Our general protocol begins with ½ unit of oxytocin; administered either subcutaneous or intramuscular, depending on the desired rate of response and duration of action. Oxytocin is only administered after eight hours of first stage labor and documented inertia. Expected results with oxytocin would be an increased frequency of the uterine contractions. Because of the short half-life of oxytocin, dosing is usually every 45-60 minutes. If the desired response of increased uterine contractility is not obtained with the first dose, doses are increased in amount until an adequate pattern of contractions is achieved. After administering three subsequent doses of oxytocin, incrementally increasing each dose, critical evaluation is made of the success of the augmentation. Failure to improve the inertia shows a strong correlation that the dystocia will not be successful utilizing a medical approach and surgical intervention is a frequent necessity.

With the presence of close-coupled contractions, a delivery should occur within one hour. Frequently when this type of contraction pattern is noted the presenting fetal part may be palpated on vaginal exam. *Note of interest*, the bitch was sleeping during this session, showing once again that subjective symptoms are not adequate markers of labor progression. Medication would be contraindicated with this uterine contraction pattern, and the first pup was delivered 15 minutes after the end of the session.

Figure 6. Contraction pattern with fetal/pelvic engagement; oxytocin/calcium contraindicated!



Fetal heart rates

Monitoring fetal heart rates in the home setting provides a window into the uterus and an opportunity to assess fetal well-being on an individual puppy basis. Using a hand-held doppler we have had excellent success teaching owners how to locate individual puppies, and follow the puppies for distress prior to or during the whelping. The range for normal fetal heart rates vary in published literature.[16,17] Our data base, in place since the mid 1990's, and being the first to monitor fetal outcomes related to heart rate, consistently documents a normal fetal heart rate range from 180-220 beats per minute (BPM). Fetal heart rates are noted to be over 200 for the most of gestation and declining to 190-200 the week before parturition. As term gestation becomes closer the rates will begin to decline into the 170-200's as a normal observation. During labor variable decelerations (a rapid decrease and return to baseline) are fairly common and not concerning unless the nadir of the drop is into the 150's or less, or the deceleration is prolonged or repeats on a frequent basis. A declining fetal heart rate baseline presents the most ominous of the concerning fetal heart rate patterns. Baseline heart rates that are running consistently less than 170, especially below 150 are associated with poor outcomes or the passage of meconium by the pup. Respiratory acidosis is associated with prolonged bradycardia, further depressing the puppy and making them more difficult to resuscitate at birth.[17,18,19]

When evaluating fetal heart rates in the veterinary office it is best to let the bitch rest/relax as adrenaline secreted in response to stress will cross the placenta and may give a false sense of fetal well-being if fetal heart rates are assessed immediately upon arrive at the clinic.

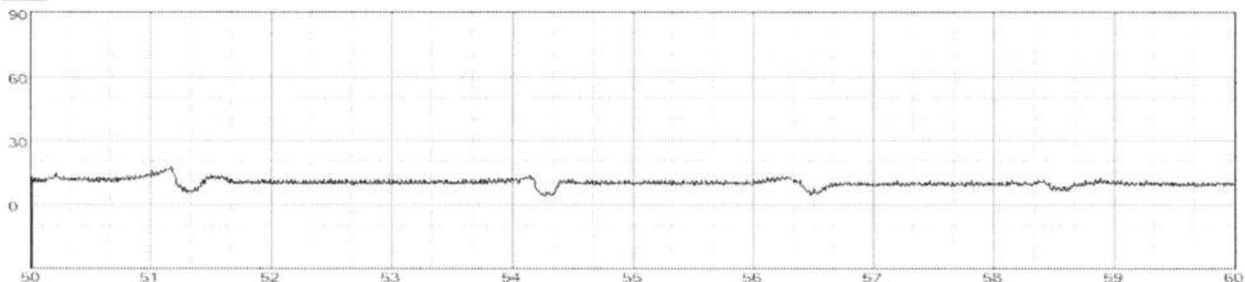
High risk pregnancy management

If you ask most canine reproduction practitioners if they feel that the incidence of mid-late pregnancy litter loss is on the increase, I believe that most would say "yes". The frustrating part of litter loss is that identifying the actual cause of the issue has been elusive. We have correlated uterine activity above the "norm" for gestational age as the primary reason for litter loss. Our overall client base consists of about 30% high-risk premature labor clients, either being monitored because of a problematic history of litter loss or acute premature labor. Documented conditions associated with the increased premature contractions have included uterine infections; both acute pyometra and low-grade metritis, hypolutealism, and uterine contractions associated with no known cause. Regardless of the cause, premature labor has been controllable in most clients. Keys to successful management have been the early documentation of uterine contraction patterns, early intervention, and medication titration to maintain uterine quiescence. Most of our high-risk clients are placed on service after either experiencing a prior litter loss, or having multiple resorptions, or other concerns being identified during the current pregnancy ultrasound. Regardless of the history at the start of service, we have successfully gotten these high-risk pregnancies to term gestation 95% of the time, including bitches that are actively delivering premature puppies. Monitoring the uterine activity and controlling aberrancies is the reason for success. High-risk management is a dynamic process; it is rare that we have a client start service under treatment "X" and not have the treatment plan modified, sometimes daily, to achieve term gestation. Developing a plan of high-risk management without knowing what the end-organ response to that plan is seems to put the practitioner at a disadvantage for prescribing effective medication protocols. Monitoring the gestation with a uterine monitor will give the practitioner an early warning that plan modification may be indicated.

Premature labor

The presence of uterine contractions in an organized fashion in the canine was first documented in 1989 by G.C van der Weyden et al, by surgically implanting electrodes in the canine myometrium.[11] van der Weyden's observation of the presence of one to three contractions an hour seven days before the onset of an active labor pattern has been strongly duplicated in our client population. We consider the occurrence of one to three contractions an hour a normal "baseline" uterine contraction pattern after 53 days after the LH surge. Uterine contractions occurring before day 53, especially with the presence of irritability (contractions that are less than a minute in length) have a high incidence of premature delivery and/or premature placental separation.

Uterine irritability at 27 days after the LH surge, prior history of losing litter around 40-45 days of gestation. Irritability is defined as uterine contractions that are less than 1 minute in duration.



Of particular concern for premature labor management is the presence of a pattern within the uterine contractions or irritability. Once a pattern of contractility is established, frequently the uterine activity will escalate into an active labor pattern within 48 hours if not treated. Clients using the WhelpWise service have a high motivation for success; most of our cases are referred to service because they have already lost litters. Our "failure" rate, or "what happens if you do not treat the contractions" has occurred from clients that are non-compliant with treatment protocols or not started on service in

time. Clients that have not been treated aggressively have gone on to lose a significant number of pups in the litter, or lose the entire litter. Compliant clients have had overall very successful outcomes.[20] It is also very important to note that rarely are there symptoms associated with premature labor, nor is premature labor associated with a decline in maternal temperature.

Hypolutealism

Progesterone decline during gestation leading to active labor and pregnancy loss has been well accepted in veterinary research.[5,14,25] Causes of hypolutealism are generally unknown, however a recent study has looked at luteal insufficiency as a consequence of an autoimmune response.[21] While it appears that many in the veterinary community want to categorize progesterone levels into good or bad based on the level, our client population shows that progesterone levels are varied and there may be a trend for a problem at a certain level, but there is not an exact measurement for a problematic progesterone level. Data collected by Sontas et al also shows a "trend" in progesterone levels, but within the study progesterone levels varied greatly.[28]

What we do not see from our database is the correlation of an "acceptable" level of progesterone for a specific point in gestation. Progesterone levels fluctuate over a fairly large range and appear to be influenced by the breed of dog and the number of whelps in the litter. Bitches on service that "should be just fine" with the current progesterone level showing preterm labor that only responds to progesterone supplementation and conversely those that have extremely low progesterone levels being very stable on the uterine monitor.

Using uterine quiescence as a marker for a "safe" progesterone level, we can use the uterine monitor to alert the practitioner that progesterone therapy is not being successful. In our high-risk client population, proactive uterine monitoring; following up with progesterone levels when the uterus is not quiet have provided early evidence that the existing progesterone level, regardless of laboratory value, is not adequate to promote a healthy uterine environment.

Infectious causes of litter loss

As documented in both human and veterinary medicine infection plays a significant role in preterm labor because of the prostaglandin F2 alpha release with resulting leutolysis.[14] The presence of infection can be difficult to ascertain and frequently high-risk bitches, especially those with a prior history of infective loss, may culture out a wide variety of resistant bacterial species. Random antibiotic use should not be considered without first doing a guarded vaginal culture to screen both for the appropriateness of antibiotic therapy as well as correct antibiotic coverage to decrease the chance of resistant microbe development. Data from our clients frequently show that a vaginal culture and subsequent antibiotic treatment on bitches that are not stable frequently benefit from the addition of antibiotic therapy.

Medications for premature labor management

Terbutaline. Brethine is one of the most frequently used tocolytic drugs in the treatment of human preterm labor. Terbutaline is in the class of drugs called beta-mimetics. These beta$_2$-adrenergic receptor agonists are sympathomimetic, causing smooth muscle relaxation by decreasing free intracellular calcium ions.[22] Controversy exists in human medicine about the long-term effectiveness of terbutaline; some of this controversy is related to the b-site saturation causing the drug to become ineffective. Titrating terbutaline doses; beginning with the smallest effective dose, proactively monitoring uterine activity, and increasing the doses in very small amounts to control uterine contractility has proven effective in human medicine.[23,24] We have seen the same clinical course with our clients. I believe that a primary reason for our success with terbutaline is that we do not begin with an arbitrary dose, but rather titrate dosing to control concerning uterine contraction patterns, increasing doses as needed.

Progesterone supplementation. In clients experiencing hypolutealism, we have seen the best response and long term stability of uterine contraction patterns when using injectable progesterone

(50mg/ml) in a carrier, usually sesame, apricot, or cottonseed oil. The oil-based medication is absorbed slower and maintains a more constant progesterone level. The efficacy of this type of therapy can also be documented through laboratory testing. Doses have ranged from 1-3mg/kg, given QOD to every fouth day, with dosing schedules determined by both laboratory values and uterine monitor results. Using the uterine monitor provides an early warning system that uterine quiescence is not being achieved with the current dose plan, allowing the veterinarian to evaluate serum levels and adjust doses if necessary. Weaning progesterone as term gestation approaches, using the uterine monitor to adjust doses, possibly add terbutaline to maintain a contraction free uterus also allows a safe taper off the progesterone; promoting a normal transition into lactation and maternal skills.

Oral progesterone. Regumate/Prometrium has not shown significant impact in the control of uterine contractions in our client population. Many clients have started on service with Regumate, but within a short period of time will need to be transitioned to the injectable. Some clients have done well with Prometra, an oral progesterone supplement from the human side, but again it seems to be the occasional client that is actually stable on the medication. While oral progesterone supplements may help improve outcomes in a non-monitored litter, frequently there is still puppy loss, or surviving pups are intrauterine growth retarded related to unhealthy placentas from uncontrolled uterine activity. Questions unanswered about the use of oral progesterone supplements and their observed lack of impact on uterine contractility would be the effect of the canine gastric pH and canine metabolic rate on the absorption and metabolism of the oral medications.

In extremely difficult cases of premature labor, the combined use of progesterone, terbutaline and antibiotics have been employed. Management of these exceptionally high-risk clients is a day-to-day observation/documentation/modification of treatments to achieve the goal of term gestation. Unfortunately, because of the multiple dynamics and the multifactorial nature of high-risk pregnancy management, no specific management technique has been successfully extracted from our client base. Each case is managed as an individual.

We do see an increased cesarean section rate for high-risk clients that have been either on progesterone supplementation or on preterm labor management. Most high-risk clients do not want to take the chance of any fetal loss, and will perform an elective cesarean section. Of those that do choose to free whelp, the incidence of fetal distress (70%) or severe inertia (20%) are risks for this group so clients are informed before the parturition date so they can discuss with their veterinarian the potential risks of a free-whelp. Close proximity to 24 hour veterinary care will also play a role in a decision to free-whelp.

Other observations from our data

The myths around progesterone

Progesterone testing has provided the canine practitioner with a wealth of information around optimal times to breed, adequate progesterone levels for pregnancy maintenance and a guide for when to perform an elective cesarean section. However, the tendency to try and consolidate data into a "cook book" format frequently encourages the practitioner to only evaluate the number and not the patient. Deviations from the progesterone "norm" that we have noted on clients on service include:

"Ovulation occurs a 5ng". We have noted that many litters, especially large litters will be at 5ng 24 hours after a positive LH, which if the bitch was to be bred two days later would put her at risk of missing completely or having a much smaller litter than her potential. Conversely, the same is true for those experiencing a slow progesterone rise. When LH timed and the rise is slow the progesterone level day 4-6 may be at 5ng or in some cases lower than 5. Overall, we see the best success for breeding when LH timing is used to time the litter, evaluating that a progesterone rise has occurred but not focusing on "ovulation occurring at 5". Also when using ovulation timing (OVT) to determine due date it is very important to know where the values are coming from. Progesterone testing is becoming more diversified with the onslaught of progesterone assay machines coming into individual practices. Calibration of these machines is critical, and while the results may say it was a "5", always compare to a well know laboratory

value such as IDEXX. Also, the incorporation of multiple laboratory sources may only add confusion to OVT so try to keep laboratory tests in the same testing laboratory if possible.

"It's safe to do a cesarean section if the progesterone is below 2ng". Veterinary literature is supportive of performing a cesarean section if the progesterone is below 3.4 "the bitch will whelp in 24 hours" or using a "2 or below" as markers that elective delivery will produce pups that are mature enough to survive.[27] "Survive", yes- "thrive", maybe not. We have had multiple clients that have been on service for preterm labor management that are having the progesterone levels monitored. While not a frequent occurrence we do see progesterone levels even at 45 days of gestation that are below 5ng, and the bitch is stable. It is not uncommon to have progesterone levels running between 2-3ng for the last seven days of pregnancy. If serial progesterone levels are not performed it is difficult to say from one progesterone level that the litter is ready to deliver. Delivering a litter early will contribute to lack of mothering skills, agalactia, and increased bleeding. A recent article from Beccaglia et al strongly suggests that multiple parameters be included in evaluation of the bitch and litter prior to an elective cesarean section.[26]

Yes- Dopram DOES work!

The most observed cause of neonatal death in our client population is hypoxia related to absent/poor placental profusion during labor, torn or ruptured umbilicus during delivery, or suppression of respirations from either metabolic acidosis or anesthetics. The hypoxic-metabolic-acidotic state creates a vicious cycle of further respiratory depression. Breaking this cycle can be difficult. Establishing respiratory effort is of paramount importance. Because of size and skill limitations, intubations/mechanical ventilation is frequently impossible, especially in the home setting. Supplemental oxygen is helpful, but only if there is respiratory effort. Over-riding the central nervous system's inability to initiate respirations has been effectively accomplished with clients through the use of Dopram.[29,30] Veterinary literature has reported mixed reviews on the effectiveness of Dopram,[32] but it has been our experience that it is very useful in stimulating respirations in apneic pups, as long as a heart rate is present.

Human literature reports that doxapram (Dopram) is effective in stimulation of the respiratory center. However, when studied in severely premature infants it has received criticism.[31] In this study Doxapram was administered to maintain respirations until the neurologic respiratory center reached maturity as these infants were born between 25-27 weeks of gestation (40 weeks for full term). Side effects noted from the use of the drug showed developmental delays, however the doxapram kept the babies breathing and off of mechanical ventilation. When evaluating the ineffectiveness of the drug it would be important to look at all aspects of prematurity, not just the possibility that doxapram caused developmental delay. Factors not evaluated by the studies were the deleterious effects of long-term mechanical ventilation having more untoward effects than did the doxapram.

For severe respiratory depression we have found the best success with administering Dopram IM, in the caudal thigh muscle, as this is usually the largest muscle mass in a pup. Injecting the drug sublingual can cause extreme pain in the surviving neonate, preventing effective nursing. For mild respiratory depression, oral administration (drops) is mildly effective, but mucosal suctioning frequently removes the drug. Dopram doses are titrated to the level of distress (presence of any respiratory effort, and neonatal heart rate). We typically suggest the IM dose of 0.01cc/4oz of neonatal weigh, watching for the usual Dopram "flush", a profound redness of the mucus membranes. This flush is positive feedback that the drug is circulating in the system and respiratory effort should soon follow. If the flush is not noted, repeated injections are done until the flush is noted. Additionally, cardiac rate is assessed and CPR performed if the pup is not stabilizing.

Whelping success

Successful management of all aspects of medicine is dependent upon objective information on which to base decisions. Management of diabetic patients require the measurement of objective blood glucose parameters to determine how to dose insulin, orthopedic problems require x-rays or an MRI to assist the veterinarian with their diagnosis. Maternal and fetal management is no different except that you

have two or more patients to manage. Using uterine and fetal heart rate monitoring will help promote successful outcomes for your clients.

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Description and analysis of gonadectomy in a cohort of Golden Retrievers

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The Golden Retriever lifetime study

The Golden Retriever Lifetime Study (GRLS) consists of approximately 3,000 privately-owned dog living through the lower 48 United States. The goal of the study is to define the incidence of and identify risk factors for hemangiosarcoma, osteosarcoma, lymphoma, and high grade mast cell tumors.

Once a year, dog owners complete and annual questionnaire that asks in-depth questions about the dog's lifestyle, environment, behavior, and nutrition. Veterinarians complete a questionnaire detailing the dog's health history and physical examination results. At the same annual appointment, routine clinical pathology is performed and blood, hair, feces, toenails, and urine are collected and banked for future research.

In addition to annual routine visits, if suspicion of malignancy exists, veterinarians are asked to complete an additional questionnaire about the dog's health as well as submit tumor tissue samples and collect the same samples as required during the routine annual visit.

We will present descriptive data about the health status of our cohort with special focus on clinically relevant reproductive data including gonadectomy rates, reported breeding activity, infertility, and reproductive diagnoses.

Gonadectomy and health outcomes

As a practicing veterinarian, counseling owners about the reproductive status of their dogs is a common and important discussion. The prevailing wisdom is that, if the owner is interested in keeping this dog as a pet, gonadectomy is indicated. This recommendation is in wide use in spite of a paucity of data regarding health risks or benefits associated with said procedure and is based largely in anecdotes and dogma.

Current veterinary research has demonstrated an association between gonadectomy and overweight and obesity (O/O) but to this point, studies have not been able to examine whether the age at which gonadectomy occurs is associated with the occurrence, timing, or severity of O/O. Because of its prospective nature, the GRLS study will be able to elucidate this important question in veterinary medicine. At this presentation, preliminary data about this association will be presented. In addition, there is conflicting evidence about the health consequences (either positive or negative) associated with gonadectomy. Data from GRLS regarding the association between gonadectomy and chronic non-traumatic orthopedic injuries will be presented.

Methods

This study was conducted using prospective data collected from the GRLS. Golden Retriever Lifetime Study is a cohort of privately-owned Golden Retrievers who live throughout the contiguous 48 states assembled to identify the incidence and risk factors for osteosarcoma, hemangiosarcoma, mast cell tumors, and lymphoma. Owner- and veterinarian- reported data and biological samples are collected annually. Because of the prospective nature of the study, we can study multiple exposures and outcomes.

To study the associations of interest, we performed survival analysis to estimate hazard ratios (HR) and 95% confidence intervals (95% CI) on the entire cohort. The outcomes of interest are the first occurrence of veterinarian-reported O/O (defined on the Purina Body Condition Score scale of >6/9) and the first occurrence of veterinarian-reported cranial cruciate ligament rupture or clinical osteoarthritis. The exposures of interest are age at gonadectomy (in four categories: < 6 months, 6 months – 1 year, > 1 year and intact) and the first diagnosis of O/O. All analyses were performed using SAS v9.2 (Sas Inc, Cary, NC).

Results

We started with 3,044 dogs at the completion of enrollment and have 84% compliance at the following annual visits. In the analysis of the association between age at gonadectomy and O/O all age categories of gonadectomy were associated with increased risk for O/O compared to intact dogs (≤ 6 months HR: 1.6, 95% CI: 1.3-2.0; 6 months to 1 year HR: 1.6, 95% CI: 1.3-1.9; > 1 year HR: 1.4, 95% CI: 1.1-1.6).

The youngest age category of gonadectomy was associated with increased risk for orthopedic injury (HR: 4.0, 95% CI: 1.8-9.0. Neither of the older age at gonadectomy categories were associated with the risk for orthopedic injury (6 months to 1 year HR: 2.0, 95% CI: 0.9-4.6; > 1 year HR: 0.8, 95% CI: 0.3-2.2). Overweight/obesity was not associated with the risk for orthopedic injury (HR: 0.97, 95% CI: 0.39-2.38).

Discussion

This study is additional evidence that earlier age at gonadectomy is associated with O/O and non-traumatic orthopedic injury and provides further evidence of the importance of counseling dog owners about the both the positive and potentially deletory outcomes associated with gonadectomy.

Interestingly, we did not find an association between O/O and the risk for orthopedic injury. However, this is a relatively young cohort and this association may become apparent as the cohort matures. In addition, we were unable to include measures of severity of orthopedic injury. It may be possible that O/O is not an early risk factor for orthopedic injury but could be associated with severity of disease.

Finally we will discuss future research plans regarding examining exposure to reproductive hormones and the risk for important chronic health outcomes such as cancer, metabolic disorders, and osteoarthritis.

Using DNA panel testing to increase genetic fitness in dogs and cats by improving genetic diversity and limiting genetic disorders

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Abstract

Many purebred dog and cat populations have limited gene pools similar to endangered exotic species in which it is critical to consider the entire population and the individuals involved in each breeding. To make breeding decisions, breeders tend to incorporate family history, phenotype assessments, and limited genetic information such as inbreeding coefficients and specific disease or trait DNA mutation tests. Unfortunately, these resources may not provide a complete overview of an animal's potential genetic contribution. We have used SNP marker sets to evaluate genomic diversity within individual dogs and breeds. In two separate studies, initial litters have shown decreased offspring homozygosity compared to their parents and in one intensively monitored breed, the Dandie Dinmont Terrier, mate selection incorporating genetic diversity resulted in increased litter sizes. Additionally, by creating species-specific panel-based genetic tests that can genotype over 175 different disorder and trait mutations in dogs and over 40 genetic disorders and traits in cats, breeders have new, easy to use, cost-effective tools to improve the health of their breeding programs. While genetic diversity or whether an animal carries a particular undesirable mutation should not be the only means of determining a desirable pairing, individual diversity and disease results should be included as factors in order to maintain the genetic health of the entire breeding population.

Introduction

The creation of a purebred dog breed, and to a lesser extent a pedigreed cat breed, by definition limits the gene pool by specifying which individuals may participate in breeding. A select group of individuals is chosen upon which to build the population. This closely resembles the situation faced in conservation genetics for endangered and threatened species where genetic diversity is extremely limited, and therefore closely monitored, as it is considered one of the fundamental levels of biodiversity. In general, genetic diversity, the variability of genes within a population, can impact both the health and long-term survival of that population since a decrease in diversity has been associated with reduced fitness. In dogs in particular, that reduction in fitness can be demonstrated as higher rates of puppy mortality (Van der Beek et al., 1999), decreased litter sizes (Van der Beek et al., 1999; Gresky et al., 2005; Calboli et al., 2008), increased incidence of inherited diseases (Janutta et al., 2008; Engelhardt et al., 2008; Donner et al., in preparation), and a decrease in life span (Long & Klei, 2014).

It is important to remember that the genetic diversity of a breed is constantly changing from generation to generation. Regardless of the number of genetic variants that are present in a breed at a point in time, the only ones that are important are the ones that are passed to the next generation and, as such, can affect future diversity. This is why genetic diversity is so easily lost and difficult to regain. Breed genetic diversity can be increased slowly through maintaining genetic diversity and allowing new mutations to develop over many generations or it can be increased quickly through an outcross to another breed.

Populations with low genetic diversity or a small population size are particularly at risk of suffering further losses in diversity. Small populations are more sensitive to the effects of selection and founder effects when only a few animals are used to start a new closed breeding group. These bottlenecks can prove to be particularly adverse to the genetic diversity of the population and without enough diversity or population size to overcome new challenges (e.g. emerging infectious diseases), a breed could be significantly impacted. Due to the nature of most companion animal populations which are spread

throughout the world, it is unlikely for a single environmental challenge to completely eliminate a breed, however there is the possibility to lose a breeding line or colony in a single event.

In the world of conservation genetics for endangered species, a species earns the "endangered" designation when there are fewer than 500 individuals which hinders efforts to avoid inbreeding. Furthermore, a "critically endangered" species is defined as a population with less than 50 genetically-unique individuals available to contribute to the next generation, also called the "effective population size" (N_e). This is the point where population genetic theory indicates that inbreeding depression will likely impact the health of the group. For comparison, a recent study estimating the inbreeding effective population size of ten breeds using pedigree information over approximately eight generations obtained from the United Kingdom Kennel Club found that eight of the breeds investigated – Akita Inu, Boxer, English bulldog, Chow chow, Rough collie, Golden retriever, German shepherd dog, and English springer spaniel – had effective population sizes of between 33 and 76 dogs which was much smaller than, but generally correlated to, the population sizes for each breed (Calboli et al., 2008). Thus, these eight populations would all be effectively considered critically endangered when evaluated by the parameters applied in conservation biology. It was also estimated that seven of the breeds whose pedigrees were studied lost over 90% of the founders genetic variants by the sixth generation demonstrating the severe effects of the breeding patterns used. The Golden retriever, in particular, showed a strong popular sire effect with 10% of the sires used producing more than 100 registered offspring; the next strongest popular sire effect was in the Labrador retriever with 5% (Calboli et al., 2008).

Based on data such as these, it is critical that we work with breeders and kennel clubs to assess their breeds and breeding animals to encourage the maintenance of genetic diversity and limit the impact of known genetic disorders. While pedigrees can be used to estimate inbreeding coefficients, there are limitations to consider. First, the inbreeding coefficient derived from a pedigree is specific to all of the offspring produced by a particular mating but cannot assess which genetic variants were inherited by individual offspring. Additionally, there are likely to be pedigree errors as estimates range up to approximately 10% of canine pedigrees contain an incorrect ancestor (Leroy et al., 2012) that could skew the results of an inbreeding coefficient calculation. Finally, the inbreeding coefficient calculation can be artificially under-estimated due to the "founder" generation in a pedigree being presumed to be genetically unrelated individuals as this is often not the case. Alternatively, using a genetic means of identifying the level of inbreeding within individual offspring eliminates the biases of pedigree analysis and provides information that can be compared equally across the breed population.

Modern molecular genetic tools can better assess the genetic diversity in any animal using cost-effective genome-wide genotyping of genetic markers (e.g. single nucleotide polymorphisms, SNPs) as shown in Figure 1. Such information is now no longer available only to researchers, but also to breeders and breed groups to be used in practical breeding programs. SNP-based genotype data can be used to:

- explore the typical level of genetic diversity/inbreeding in a breed by measuring heterozygosity (percentage of alleles that are different at each marker) or homozygosity (percentage of alleles that are the same at each marker)
- monitor the change in genetic diversity levels over generations
- identify which matings optimally increase genetic diversity in the offspring
- visualize genetic relationships and population substructure within the breed or population

In conjunction with the creation of genetic marker panels for diversity, it is also possible to incorporate known genetic disease and trait mutations so that potential breeding animals can be rapidly and efficiently screened to inform the breeder of any concerns prior to mate selection. This screening can be performed at any age so it can also be used to determine which offspring of a desirable mating can be retained for future inclusion in the breeding program.

Ultimately, lack of genetic diversity can risk a breed's sustainability and therefore, we need to equip breeders with new genetic tools that can consider the entire breed population, as well as the

individuals involved in any given breeding, to preserve their breed. This study demonstrates, as a proof of concept, the use of two different SNP-based genetic panels used to calculate genetic diversity and inform breeders for mate selection in order to improve genetic diversity in the offspring and improve other measures of fitness.

Materials and methods

Blood, cheek swab, or semen samples were obtained from purebred dogs of various breeds and their DNA was typed on one of two custom SNP panels (Optimal Selection™ by Mars Veterinary, Vancouver, WA or MyDogDNA® Breeder by Genoscoper Laboratories, Helsinki, Finland) using either the Sequenom platform (Sequenom, Inc., San Diego, CA) or the Illumina HD Ultra platform (Illumina, Inc., San Diego, CA), respectively, following standard manufacturer protocols. Any low quality samples were discarded and retests offered. DNA data for each panel were analyzed for genetic diversity through either their percentage of homozygosity in Optimal Selection™ or heterozygosity (1-homozygosity) in MyDogDNA®. Breeder and prospective matings were scored based on the diversity that could be achieved in the expected litter. Results were then presented to breeders in the form of a Breeding Score or Genetic Health Index, respectively.

For the intensive study of the Dandie Dinmont Terrier (DDT) breed, over 250 DDTs in the U.S. (>90% of the potential breeding population) were analyzed using Optimal Selection. Breeders incorporated this genetic diversity data into their mate selection process and the resulting litters were evaluated for the number of puppies born and their genetic diversity through homozygosity levels. Statistical analyses were performed using a two-tail t-test assuming unequal variance. Their long-term health is also being monitored.

To date, a combined reference database has been built using a population of over 20,000 purebred dogs representing more than 300 breeds.

Results

Heterozygosity and homozygosity levels were determined for each breed analyzed, an example of which is shown in Figure 2a which illustrates the utility of and information gained by comprehensive SNP-based genotyping. As shown, on average, the evaluated Labrador retrievers are slightly more genetically diverse than purebred dogs as a whole, however, they are clearly less diverse than the mixed breed population. A particular dog's heterozygosity can also be presented on the graph in comparison to the rest of the breed. Population substructure can also visualized on heterozygosity graphs in some cases. In Golden retrievers, a subgroup of dogs with a higher heterozygosity can be identified (Figure 2b). This group represents working line dogs, supporting the observed divergence between working and show/companion Golden retriever populations. Similar evaluation of genetic heterozygosity can be used to monitor the levels of diversity over time. As an example, the Finnish Kromfohrländer breed club has been actively outcrossing to other breeds – Tibetan Terrier, Parson Russell Terrier, and Poodle (Medium variety by the FCI size standard) – to revive the breed's genetic diversity. The heterozygosity levels of the two subpopulations (original Kromfohrländer dogs in the left peak and the Kromfohrländer outcross population in the right peak) as shown in Figure 2c provides a concrete view of the progress that has been made in increasing the breed's mean heterozygosity.

In the US DDT population, DNA from the resulting puppies was evaluated to determine if there was improvement in the genetic diversity. Nineteen puppies from four Optimal Selection DDT litters have been genetically evaluated and have shown an overall decrease in their average homozygosity compared to their parents, although it does not quite reach statistical significance (71.7% vs. 75.4%, P=0.053).

Specific litter data from the MyDogDNA Breeder evaluation show a similar trend towards an improvement in puppy heterozygosity compared to their dam and sire (Table). Importantly, knowledge of the litter variation and individual differences in heterozygosity between the puppies enables gene pool-maintaining selection of future breeding animals.

While it can be difficult to obtain historical birth rates for the DDT breed, the 2010 American Kennel Club registration rate for the DDT was only 2.11 pups/litter (38 registered puppies in 18 litters) and the DDT Club of America reports the historical breed average registration rate is approximately 2.75 puppies/litter (Miriam Couto, personal communication). By comparison, Optimal Selection data were available for 23 DDT matings through 2013 with 83 puppies born, indicating an average of 3.6 puppies/litter (range 1-6). The type of breeding/insemination performed varied among the matings. Natural breeding (4), artificial insemination (AI) with fresh semen (3), AI with fresh chilled semen (4), AI with frozen semen (2), surgical implantation of fresh chilled semen (1), and surgical implantation of frozen semen (9) (Figure 3) were all used to varying degrees which likely also had an impact on litter size.

Additionally, SNP-type genomic information can be used to better understand the breed's ancestry, population substructure, and the genetic relationships of animals to one another. As an example, differences due to geography and breeding line divergence can be visualized in dog breeds as shown in Figure 4 for the Norwegian elkhound breed and in Figure 5 for the German shepherd dog breed group including the longhaired and White Swiss shepherd subpopulations.

Discussion

Limited diversity within and across breeds can have an impact on the breed's overall health and reproductive well-being. This study has sought to demonstrate that in any litter, you will find variation in genetic diversity in the form of heterozygosity between the siblings. Thus, using these types of whole-genome assessment tools can help breeders capture, understand, and leverage the genetic diversity within their breed to maintain, and perhaps increase, allelic variety in future generations. Based on these data, using these tests in litter planning is:

- Increasing puppies/litter average compared to recent breed statistics
- Positively impacting the average puppy diversity

- Allowing breeders to observe substructure in their lines compared to the breed as a whole due to factors like geography, phenotype, and intended use (work vs show vs companion)

It is entirely possible to work with the genetic diversity present in any given population or breed to maintain the genetic diversity on any level, be it the entire breed, the breed within a certain geography, or within a single breeder's family line and thereby have a positive impact on the fitness of the animals involved.

Additionally, the use of panel-based genetic testing for hundreds of disease and trait associated mutations, in combination with the diversity markers, can further assist breeders with appropriate mate selection. This method of testing has also been able to identify when disease mutations thought to affect only a narrow range of breeds, may in fact affect additional breeds that can be important information for breeders and pet parents to have. For example a recent study by Donner et al. (2016) examining nearly 100 disorders in approximately 7,000 purebred dogs found the presence of several disorders in dog breeds not previously known to carry the mutations. It is important to remember that not every disease mutation will be clinically relevant in every breed so additional clinical follow-up is required to establish a causal link which this study was able to do for several of the new breeds identified. This type of widespread mutation testing also allows for unbiased assessment of mutation allele frequencies in breeds and subpopulations that can be extremely informative to researchers and breeders. Similar type population studies in even larger cohorts of pure and mixed-breed dogs, as well as for cat populations, are currently underway.

Building on the increasing number of identified disease mutations that can clinically affect individual breeds, obtaining informed and understandable genetic information and counselling for veterinary professionals as well as breeders is increasingly important. Thus, it is imperative that clear information and tools are provided to assist in mate selection that makes use of all the data available or in disease diagnosis should an animal be affected by a genetic condition.

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Additionally, the use of panel-based genetic testing for hundreds of disease and trait associated mutations, in combination with the diversity markers, can further assist breeders with appropriate mate selection. This method of testing has also been able to identify when disease mutations thought to affect only a narrow range of breeds, may in fact affect additional breeds that can be important information for breeders and pet parents to have. For example a recent study by Donner et al. (2016) examining nearly 100 disorders in approximately 7,000 purebred dogs found the presence of several disorders in dog breeds not previously known to carry the mutations. It is important to remember that not every disease mutation will be clinically relevant in every breed so additional clinical follow-up is required to establish a causal link which this study was able to do for several of the new breeds identified. This type of widespread mutation testing also allows for unbiased assessment of mutation allele frequencies in breeds and subpopulations that can be extremely informative to researchers and breeders. Similar type population studies in even larger cohorts of pure and mixed-breed dogs, as well as for cat populations, are currently underway.

Building on the increasing number of identified disease mutations that can clinically affect individual breeds, obtaining informed and understandable genetic information and counselling for veterinary professionals as well as breeders is increasingly important. Thus, it is imperative that clear information and tools are provided to assist in mate selection that makes use of all the data available or in disease diagnosis should an animal be affected by a genetic condition.

individuals involved in any given breeding, to preserve their breed. This study demonstrates, as a proof of concept, the use of two different SNP-based genetic panels used to calculate genetic diversity and inform breeders for mate selection in order to improve genetic diversity in the offspring and improve other measures of fitness.

Materials and methods

Blood, cheek swab, or semen samples were obtained from purebred dogs of various breeds and their DNA was typed on one of two custom SNP panels (Optimal Selection™ by Mars Veterinary, Vancouver, WA or MyDogDNA® Breeder by Genoscoper Laboratories, Helsinki, Finland) using either the Sequenom platform (Sequenom, Inc., San Diego, CA) or the Illumina HD Ultra platform (Illumina, Inc., San Diego, CA), respectively, following standard manufacturer protocols. Any low quality samples were discarded and retests offered. DNA data for each panel were analyzed for genetic diversity through either their percentage of homozygosity in Optimal Selection™ or heterozygosity (1-homozygosity) in MyDogDNA®. Breeder and prospective matings were scored based on the diversity that could be achieved in the expected litter. Results were then presented to breeders in the form of a Breeding Score or Genetic Health Index, respectively.

For the intensive study of the Dandie Dinmont Terrier (DDT) breed, over 250 DDTs in the U.S. (>90% of the potential breeding population) were analyzed using Optimal Selection. Breeders incorporated this genetic diversity data into their mate selection process and the resulting litters were evaluated for the number of puppies born and their genetic diversity through homozygosity levels. Statistical analyses were performed using a two-tail t-test assuming unequal variance. Their long-term health is also being monitored.

To date, a combined reference database has been built using a population of over 20,000 purebred dogs representing more than 300 breeds.

Results

Heterozygosity and homozygosity levels were determined for each breed analyzed, an example of which is shown in Figure 2a which illustrates the utility of and information gained by comprehensive SNP-based genotyping. As shown, on average, the evaluated Labrador retrievers are slightly more genetically diverse than purebred dogs as a whole, however, they are clearly less diverse than the mixed breed population. A particular dog's heterozygosity can also be presented on the graph in comparison to the rest of the breed. Population substructure can also visualized on heterozygosity graphs in some cases. In Golden retrievers, a subgroup of dogs with a higher heterozygosity can be identified (Figure 2b). This group represents working line dogs, supporting the observed divergence between working and show/companion Golden retriever populations. Similar evaluation of genetic heterozygosity can be used to monitor the levels of diversity over time. As an example, the Finnish Kromfohrländer breed club has been actively outcrossing to other breeds – Tibetan Terrier, Parson Russell Terrier, and Poodle (Medium variety by the FCI size standard) – to revive the breed's genetic diversity. The heterozygosity levels of the two subpopulations (original Kromfohrländer dogs in the left peak and the Kromfohrländer outcross population in the right peak) as shown in Figure 2c provides a concrete view of the progress that has been made in increasing the breed's mean heterozygosity.

In the US DDT population, DNA from the resulting puppies was evaluated to determine if there was improvement in the genetic diversity. Nineteen puppies from four Optimal Selection DDT litters have been genetically evaluated and have shown an overall decrease in their average homozygosity compared to their parents, although it does not quite reach statistical significance (71.7% vs. 75.4%, $P=0.053$).

Specific litter data from the MyDogDNA Breeder evaluation show a similar trend towards an improvement in puppy heterozygosity compared to their dam and sire (Table). Importantly, knowledge of the litter variation and individual differences in heterozygosity between the puppies enables gene pool-maintaining selection of future breeding animals.

While it can be difficult to obtain historical birth rates for the DDT breed, the 2010 American Kennel Club registration rate for the DDT was only 2.11 pups/litter (38 registered puppies in 18 litters) and the DDT Club of America reports the historical breed average registration rate is approximately 2.75 puppies/litter (Miriam Couto, personal communication). By comparison, Optimal Selection data were available for 23 DDT matings through 2013 with 83 puppies born, indicating an average of 3.6 puppies/litter (range 1-6). The type of breeding/insemination performed varied among the matings. Natural breeding (4), artificial insemination (AI) with fresh semen (3), AI with fresh chilled semen (4), AI with frozen semen (2), surgical implantation of fresh chilled semen (1), and surgical implantation of frozen semen (9) (Figure 3) were all used to varying degrees which likely also had an impact on litter size.

Additionally, SNP-type genomic information can be used to better understand the breed's ancestry, population substructure, and the genetic relationships of animals to one another. As an example, differences due to geography and breeding line divergence can be visualized in dog breeds as shown in Figure 4 for the Norwegian elkhound breed and in Figure 5 for the German shepherd dog breed group including the longhaired and White Swiss shepherd subpopulations.

Discussion

Limited diversity within and across breeds can have an impact on the breed's overall health and reproductive well-being. This study has sought to demonstrate that in any litter, you will find variation in genetic diversity in the form of heterozygosity between the siblings. Thus, using these types of whole-genome assessment tools can help breeders capture, understand, and leverage the genetic diversity within their breed to maintain, and perhaps increase, allelic variety in future generations. Based on these data, using these tests in litter planning is:

- Increasing puppies/litter average compared to recent breed statistics
- Positively impacting the average puppy diversity

- Allowing breeders to observe substructure in their lines compared to the breed as a whole due to factors like geography, phenotype, and intended use (work vs show vs companion)

It is entirely possible to work with the genetic diversity present in any given population or breed to maintain the genetic diversity on any level, be it the entire breed, the breed within a certain geography, or within a single breeder's family line and thereby have a positive impact on the fitness of the animals involved.

Additionally, the use of panel-based genetic testing for hundreds of disease and trait associated mutations, in combination with the diversity markers, can further assist breeders with appropriate mate selection. This method of testing has also been able to identify when disease mutations thought to affect only a narrow range of breeds, may in fact affect additional breeds that can be important information for breeders and pet parents to have. For example a recent study by Donner et al. (2016) examining nearly 100 disorders in approximately 7,000 purebred dogs found the presence of several disorders in dog breeds not previously known to carry the mutations. It is important to remember that not every disease mutation will be clinically relevant in every breed so additional clinical follow-up is required to establish a causal link which this study was able to do for several of the new breeds identified. This type of widespread mutation testing also allows for unbiased assessment of mutation allele frequencies in breeds and subpopulations that can be extremely informative to researchers and breeders. Similar type population studies in even larger cohorts of pure and mixed-breed dogs, as well as for cat populations, are currently underway.

Building on the increasing number of identified disease mutations that can clinically affect individual breeds, obtaining informed and understandable genetic information and counselling for veterinary professionals as well as breeders is increasingly important. Thus, it is imperative that clear information and tools are provided to assist in mate selection that makes use of all the data available or in disease diagnosis should an animal be affected by a genetic condition.

In conclusion, while genetic diversity and disease mutation results should not be the only means of determining a desirable mating, the genetic assessment of the individuals should be included as a factor to maintain the genetic health of the entire breeding population. Breed health and welfare is ultimately best promoted by a holistic view to breeding, taking all areas of information into consideration including health information from hip/eye examination, physical and behavioral characteristics, tests and trial scores if we are to ensure that our dog and cat breeds can survive and thrive for generations to come. Sustainable breeding should also involve thoughtful consideration of inherited disorders and genetic diversity and panel testing can be used to further these goals.

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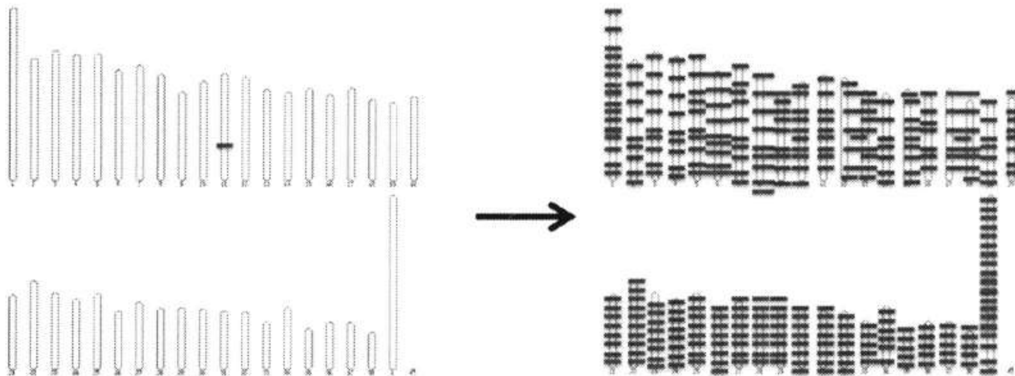

Figure 1. Transition of canine genetic testing from single mutation in the genome to genome-wide testing of thousands of mutations at once.

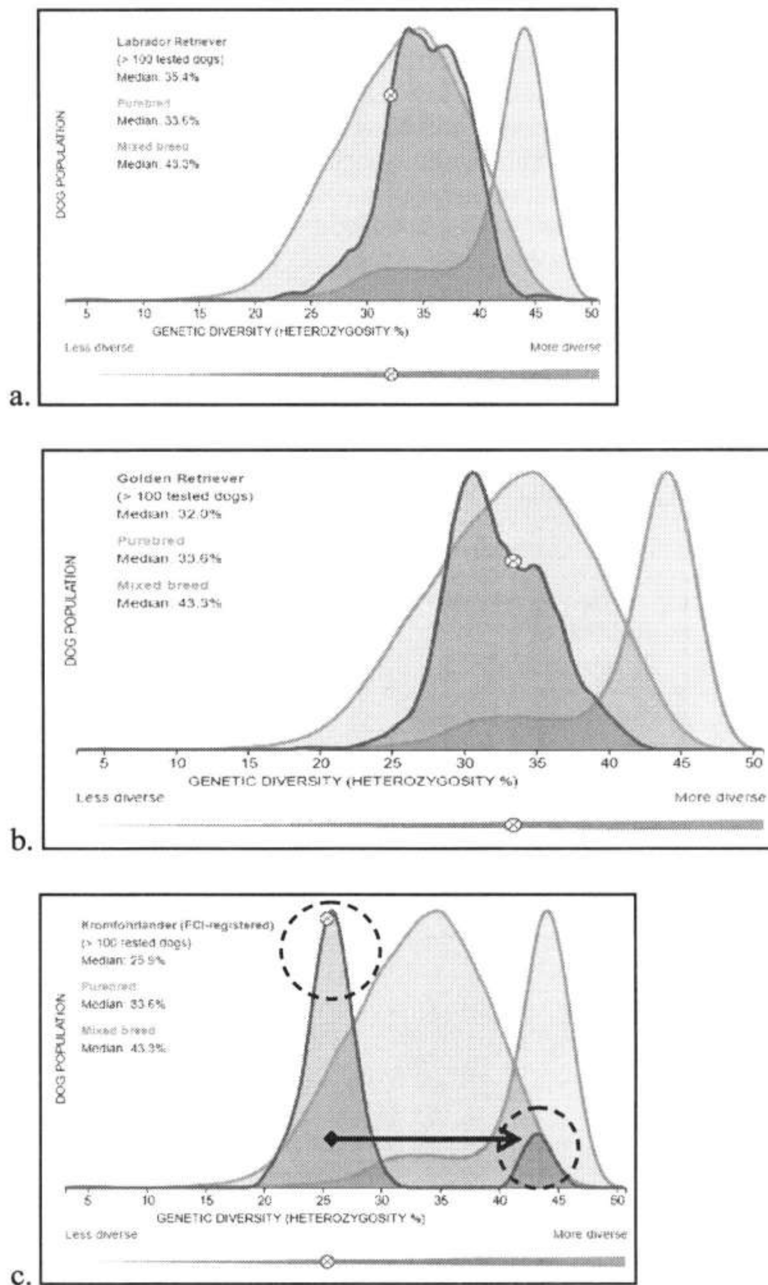

Figure 2. Measurement and visualization of heterozygosity levels. (a) Labrador retriever heterozygosity as compared to all pure and mixed breed dogs tested using a SNP-panel based measure of genetic diversity. A single Labrador retriever's data are presented as an open circle on the graph. (b) Golden retrievers as compared to all pure and mixed breed dogs tested. Note the shoulder on the right side of the Golden Retriever curve representing working lines with more genetic diversity. (c) Heterozygosity of two subpopulations of Kromfohrländer dogs – purebred dogs in the left peak and the Kromfohrländer outcross population in the right peak – as compared to the entire purebred population tested (large middle peak) and the mixed breed population tested (large right peak).

Litter 1

	Heterozygosity	Range
Dam	0.293	
Sire	0.313	
Litter average (6 pups)	0.304	0.286-0.317

Litter 2

	Heterozygosity	Range
Dam	0.295	
Sire	0.313	
Litter average (5 pups)	0.318	0.304-0.329

Table. Average heterozygosity and range in two litters of Lagotto Romagnolo puppies compared to heterozygosity of their sire and dam.

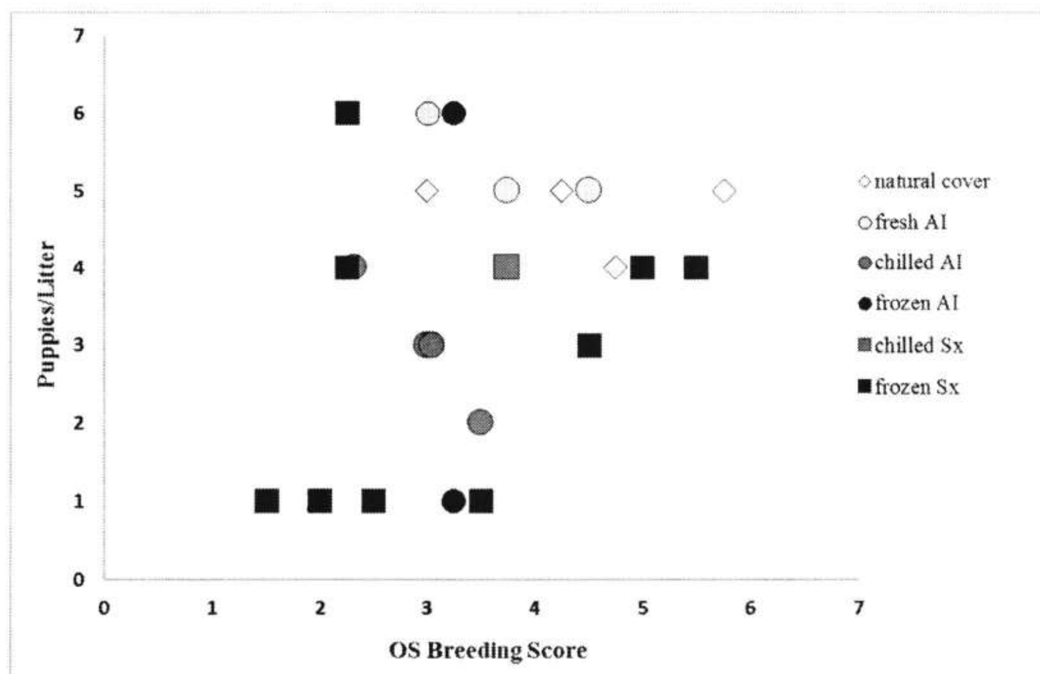

Figure 3. Comparison of Optimal Selection breeding score (lower score predicts improved genetic diversity in the offspring) and litter size with insemination technique for 23 litters of Dandie Dinmont Terriers.

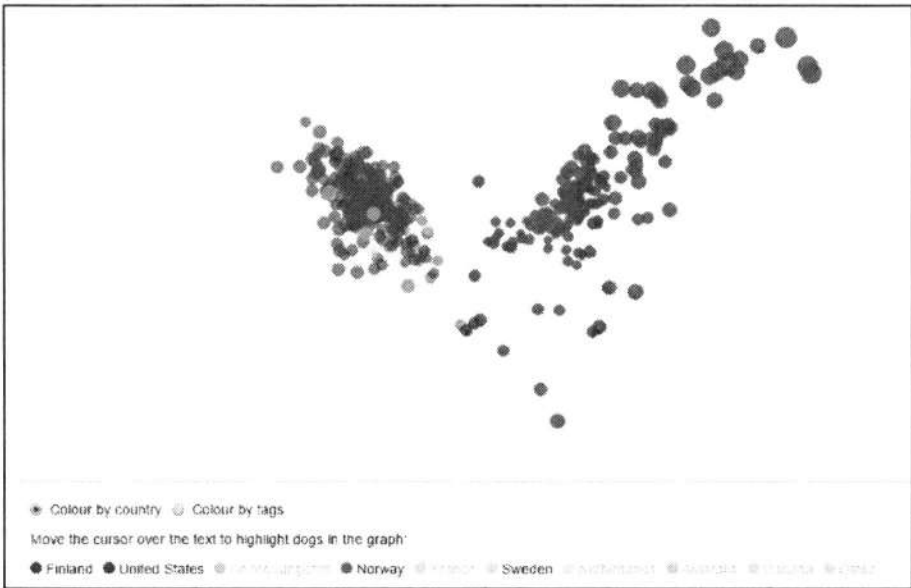

Figure 4. Principle component analysis (PCA) demonstrating geographical divergence in Norwegian Elkhounds in the Nordic countries (cluster on the left side) versus the United States (cluster on the right side). Principle component 1 (PC1) which accounts for the greatest variance between samples is plotted on the X-axis and PC2 which accounts for the second greatest variance is plotted on the Y-axis.

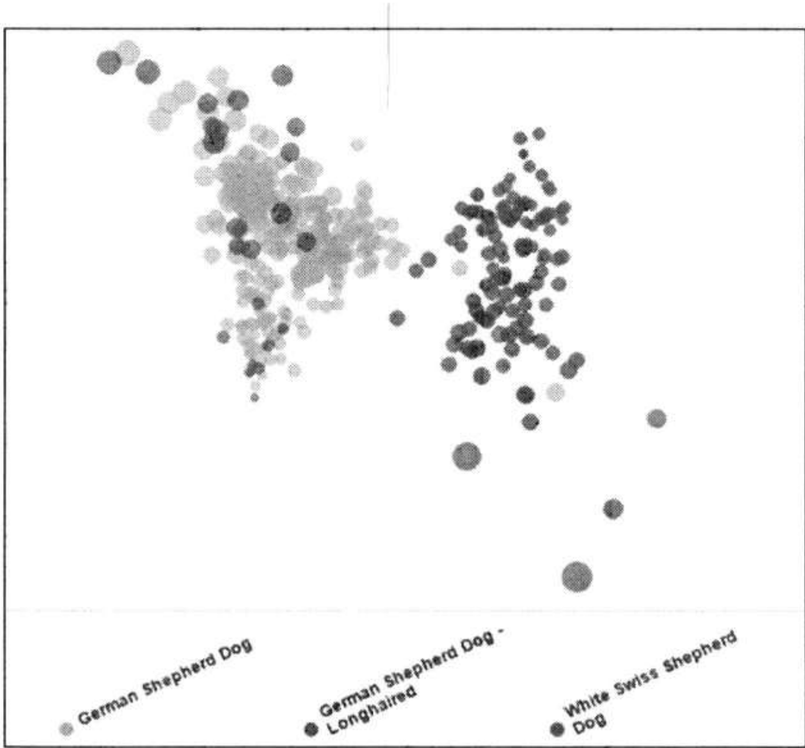

Figure 5. Principle component analysis (PCA) demonstrating the genetic relationships and substructure in the German Shepherd Dog breed group including the Longhaired variety and the White Swiss Shepherd. The German Shepherd samples are noted in light grey (left cluster) while the White Swiss Shepherd samples are noted in dark grey (right cluster) with samples from the Longhaired German Shepherd variety represented in medium grey and cover both clusters.

Swine theriogenology: what you need to know

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Background

Thousands of small farms raise a few pigs, along with an assortment of other livestock. More significant is the number of 4-H and FFA youth who raise a pig or two to exhibit at county and state fairs and winter jackpot shows. Competition in these events is often intense, so when things go awry with a show pig, veterinarians are often called to the rescue.

Many of the veterinarians who receive these calls specialize in companion animal care. They have little field experience in medical management of pigs. This paper is written for these veterinarians and focuses on one aspect of swine health – reproduction.

But first, a word on vaccinations

Vaccinations are essential to all aspects of swine health, including reproduction. Recommended vaccinations depend upon the circumstances of a swine operation. For "closed" facilities, with no movement of pigs in or out, a minimal selection of vaccinations for reproductive performance may suffice. This should include a parvo-lepto combination. Location also affects vaccination choice. In many areas of the U.S., vaccinations for porcine reproductive and respiratory syndrome (PRRS) must be included as well as others such as circovirus and *Mycoplasma* sp. A resource for this information is the 10th edition of *Diseases of swine* (Zimmerman et.al.) or a field primer is *Doc sanders goes whole hog*!

Puberty and estrus

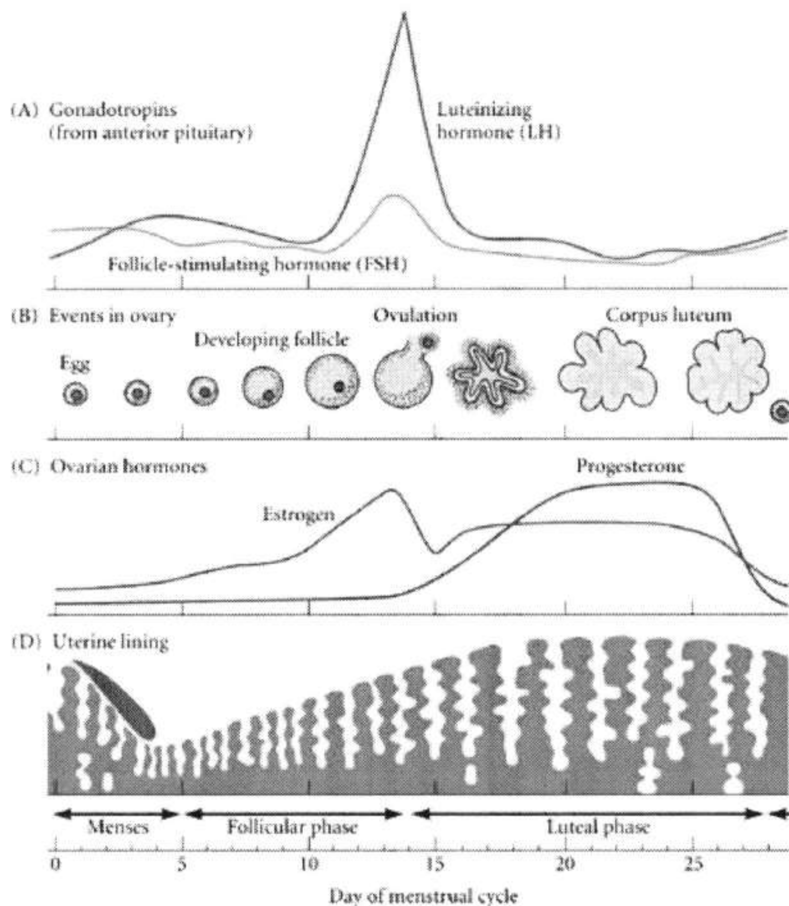
Gilts of European swine breeds enter puberty at about 25 weeks. Yorkshire breed females often require a couple months more. Factors affecting puberty include good nutrition, body weight of 120 kg., and exposure to a boar.

The porcine estrous cycle lasts 21 days – the same as for bovines. However, unlike the bovine, porcine females do not have a biphasic or diphasic follicular wave. As a gilt approaches puberty, hormone regulation occurs with a decrease of an estradiol negative feedback on gonadotropin releasing hormone (GnRH) synthesis with subsequent increase in both luteinizing hormone (LH) and follicle stimulating hormone (FSH).[1] The number of antral follicles increases as estrus approaches.

Most consider observation an effective way to detect the onset of estrus and when a sow should be bred. Observation of a swollen, reddened vulva is often a useful anatomical indicator for a gilt in heat. Note this caution: it is also possible to have estrogenic mycotoxins causing prepuberal vulvar development prior to reaching puberty.

An effective way to induce estrus in gilts is to take them on a stressful trailer ride for a few miles, followed by placing them in a pen across the fence from a boar. This takes advantage of pheromones from the boar, which play an important role in estrus and ovulation. Most gilts treated to this technique will be in estrus in five to seven days. Other options for inducing estrus include:

- A luteotrophic drug, PG 600™, which brings mature sows into heat when the farm operator wants estrus to begin on a specific date.
- Oral altrenogest products – Matrix™ and Altresyn™. After one or the other of these products is incorporated into a sow's diet for a couple of weeks, the product is removed from the diet, sending the sow into estrus.
- ProstaglandinF2α (PGF2α) administered to sows two weeks after they are bred. The treated sows will absorb their pregnancy and appear in fertile estrus in 72 hours. This program is useful in embryo transfer programs as it precisely synchronizes estrus of donor sows and recipient sows.



Hormone profiles of the sow estrus cycle.[2]

Diestrus refers to the luteal phase after the proestrus and estrus period. Signs of proestrus include a swollen reddened vulva, sometimes with a slight amount of mucus at the vulvar lips. Anatomical signs of estrus in sows are similar to that of other species, but in swine, the female's behavioral changes are very useful. The sow seeks out the boar, contrary to most mammals in which the male seeks out the female.

Sows exhibit a characteristic stance in the presence of a boar. Their ears stand erect as they assume an immovable four-point stance, even when prodded. When breeding with an inexperienced boar, coaching and guidance are required, to line up the sow and boar for coitus.

Breeding

A sow will cycle back into heat within five to seven days after weaning her pigs, unless she is a "heavy milker" and loses significant body condition. Such a sow requires more time before her estrous cycle will start again. Sows are often weaned in groups.

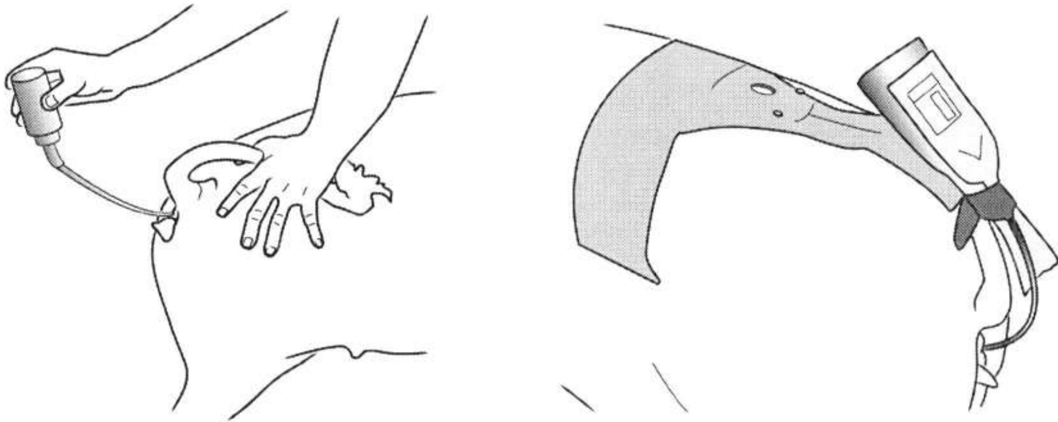
Sows are usually in heat for 40 to 48 hours. Breeding twice, at 12 and 24 hours during this period, works best. This typically results in larger litters. Ovulation starts 36 to 44 hours after the LH surge, just prior to signs of estrus. While ovulation often occurs in two to four hours, the range can last from one to nine hours.

If natural service is used, a boar is typically required for each six sows. When "boar power" is lacking, litter sizes tend to be smaller in later breedings.

With the advent of successful artificial insemination (AI) protocols, small operations can implement AI protocols similar to commercial operation management practices.

As previously mentioned, the presence of a boar is extremely effective in stimulating estrus and ovulation. Large operations keep a boar or two on premises, running them up and down the aisles to circulate their pheromones. Unable to afford this luxury, small farms can substitute the natural pheromones of boars with a spray product, Hog Mate, to prepare sows for breeding by AI.

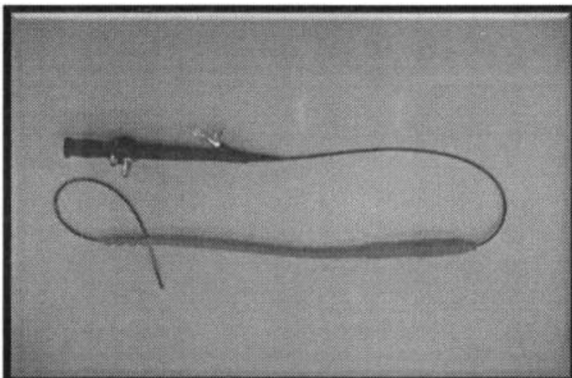
Artificial insemination

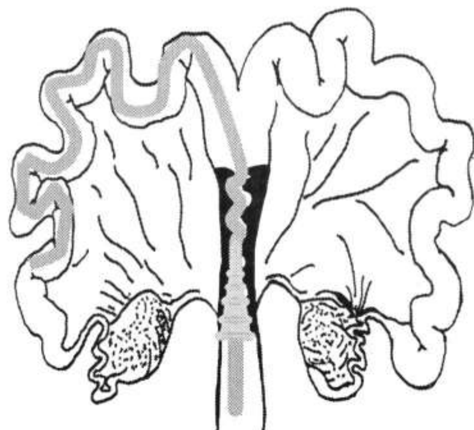


During insemination, downward pressure is put on the sow's loin. Insemination may take up to 20 minutes. The technician must be patient. A rushed insemination results in reduced litter size, and in some cases, no pregnancy at all.

Most people are surprised that nearly 85 to 90% of swine breeding in commercial operations is by AI. Four distinct AI protocols are available:

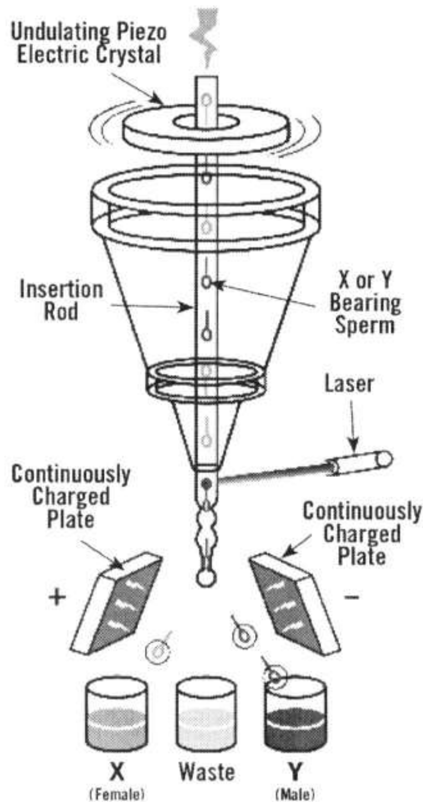
1. Frozen semen, which impregnates a sow, but reduces litter size by as much as a third.
2. Fresh, chilled, extended semen inseminated into the cervix via an AI catheter which greatly improves the litter size over using frozen semen.
3. Fresh, chilled, extended semen inseminated caudal to the cervix. This technique has become popular because it results in a pig or two more per litter.
4. Fresh, chilled, extended semen introduced by deep horn insemination near each Fallopian tube. This protocol has been developed but not yet implemented outside research and development facilities. The technique, which greatly increases litter size, is being refined for easy use by technicians. Someday technicians will be able to fertilize over 40 ova in a sow via deep horn insemination. The question is how to manage this many embryos, as a sow's uterus runs out of room at half this number. The technique also requires a much smaller insemination dose. This suggests that only the top genetic-merit boars would be used – by some predictions, only the top two percent.





A special adapted endoscope is used to thread the catheter near the Fallopian tube via fluoroscope. Utilizing this method the sperm dose can successfully be reduced to 2% of a normal 50 million sperm dose.

Yet another option – sexed semen – is being developed for swine, using similar technology developed for routine use in cattle. A genetics company is in the final stages of releasing this option in the swine genetics marketplace.



Sexed semen for swine is in final stages of development using flow cytometry. (*Courtesy of Sexing Technologies, Navasota, Texas)*

Pregnancy examination

Several methods are used to detect pregnancy in a sow. Real time B ultrasound examinations are the best option. Other choices, such as observing return to estrus in 21 days, lead to many missed non-pregnant sows. Estrone blood tests are considered too cumbersome for small commercial operations.

Farrowing

The old cliché "three months, three weeks and three days" pretty much predicts the swine gestation period of 114 days. But the exact time of a farrowing cannot be predicted with accuracy. This means farrowing can occur unsupervised, especially when farm management is spread thin or the owner has another job. A useful technique for ensuring adequate supervision is to induce farrowing at 110-112 days with PGF2α + oxytocin. Supervised farrowing results in fewer stillborn pigs, according to available data. Small doses of oxytocin are also very useful during farrowing when a sow is becoming noticeably fatigued. Managed obstetrics is often prevents the need for a cesarean section. Confirm the due date and verify that the sow has milk in her mammary glands. Milk usually can be stripped out of the teats in the last 12 hours prior to farrowing.

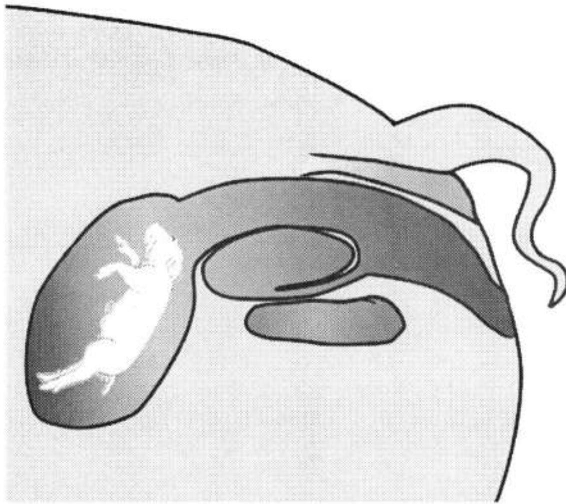
Obstetrics

Maternal-fetal mismatch – that is, piglets too large for the birth canal – is often the primary reason for dystocia in a sow. This situation is more common for first-litter gilts. When a sow carries only three or four pigs to term, the pigs are usually larger than normal.

A cesarian section is usually a salvage operation for these and other incongruities that occur in the farrowing process. Generally, cesarean sections are most successful when initiated early in the course of parturition. This reduces risk to the health of the sow and her babies.

If pigs are slow to be delivered, an aseptic vaginal and pelvic examination should be performed to determine what is delaying delivery. Due to risk of infection of the sow and piglets, bare arm examinations are not appropriate, nor is examination without a thorough cleaning of the vulva and perineal area.

A pelvic examination will help determine whether a sow's pelvis is too narrow or the pigs are large relative to the size of the birth canal. In rare cases, a mal-positioned pig in the birth canal causes dystocia; for instance, a piglet positioned upside down. The geometry of the pelvic canal, 60 to 90 degrees opposite that of the pig, prevents any opportunity to deliver without turning the pig over 180 degrees.



A pig positioned dorsal-ventral has a low probability of delivery and survival because of the anatomic contradiction of sow birth canal anatomy and the pig's rigid dorsum. – *Doc Sanders Goes Whole Hog, Vaca Resources. Urbana, Ohio, 2015.*

Cesarian section is often recommended when dystocia is obvious or when the owner reports that the piglets are extremely valuable. Nothing is more discouraging than delivering a litter of dead pigs. The following are examples of sow anesthesia options that are useful in cesarean sections, depending on the judgment and experience of the surgeon:

1. Reconstitute 1 vial of telazol with 2.5 ml. xylazine (100 mg/ml) and ketamine (100 mg/ml). Give 1 ml/50 lb IM. (G. Hubbell, Ohio State CVM)
2. Reconstitute 1 vial of telazol with 5 ml xylazine (100 mg/ml). Give 1 ml/50 lb IM. (F. Welker, Ohio State CVM)
3. Xylazine (100 mg/ml), ketamine (5 mg/lb), and butorphanol (0.1 mg/lb). Give IM. (F. Welker, Ohio State CVM)
4. Xylazine (100 mg/ml) IM. Mask halothane anesthesia to effect. (D. Sanders, UVC)
5. Dexmedetomidine (0.02 mg/kg) with ketamine (5 mg/kg) and butorphanol (0.2 mg/kg) IM. (Anesthesia and analgesia for veterinary technicians. 4th ed. p. 290, Thomas and Lerche)

The preferred site for a cesarean section incision is a few inches above and parallel to the mammary glands. Surgical preperation is essential for the well-being and comfort of the sow. The surgical site should be draped.

The uterine horns of a mature sow may be a meter or more long, each loaded with pigs. Often, each horn must be surgically opened to locate and remove every pig. Gloves should be rinsed in heparinized saline before entering the abdominal cavity. Hemostats should be used for hemorrhage control, rather than 4 X 4 gauze sponges. Sponges tend to create locations for adhesions to develop after surgery. Adhesions may not be critical if the sow is leaving the herd after surgery but use of gauze sponges should be avoided if future surgery is anticipated for a sow.

At least a couple of attendants should be on hand to take and revive pigs as they are removed from the uterine horns. A pitcher of 105^0F water is very helpful for reviving newborn pigs. Insert each pig in the water up to its head. After they are revived in this way, they should be dried, the umbilicus should be clipped down to a couple of centimeters, and the stump should be dipped in two percent iodine or an equivalent disinfectant.

Each pig should be provided colostrum within a couple hours of birth. Stripping colostrum from the sow's teats and administering it to each pig is very beneficial. If farrowing management is a significant part of a clinic's work, additional colostrum should be collected from mature sows with a prodigious supply. This colostrum can be frozen in an ice cube trays so that a cube at a time can be thawed as needed for a low-vitality pig or to make up for a sow with inadequate colostrum. The colostrum should be administered by stomach tube, similar to administration in newborn puppies.

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3. Sanders DE: Doc Sanders goes whole hog, Urbana(OH): Vaca Resources; 2015; p. 133.

Reproductive management of reindeer (*Rangifer tarandus*)

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Introduction

Reindeer (*Rangifer tarandus tarandus*) and caribou (*R. t. granti*), are relatively recent additions to the deer family. Both are members of the same species, which is further subdivided into two main groups, with seven extant subspecies, all being indigenous to holartic northern latitudes.[1,2]

Reindeer and caribou are unique among cervids because both sexes bear antlers, suggesting that antler development is largely independent of gonadal activity.

Reindeer have been domesticated for centuries, and provide an economic mainstay for many native populations. In North America, they are primarily kept for meat production and seasonal exhibition. As with other domestic species, owners strive to increase reproductive efficiency.

Because reindeer are managed very extensively, environmental factors interact significantly with fecundity. Current evidence suggests that management of these factors - of which nutrition, reproduction, and herd composition seem to be the most important - will enable the productivity of reindeer herds to be significantly improved. A recent move towards a more intense production of reindeer, using traditional agricultural methods, requires intensified management and enhanced understanding of reindeer reproductive management. A review of both female and male reproductive management will be presented as a guide for practicing veterinarians to understand and address the peculiarities of this species.

Female reproductive management

Estrous cycle

Reindeer, like most cervids, are seasonally polyestrus, with an estrous cycle length of approximately 24 ± 3.4 days in North American reindeer.[3] Considerable variation has been reported in primiparous Norwegian reindeer with an average estrous cycle length of 19.4 ± 5.7 days.[4] Seasonal ovarian activity is initiated in late August. As in other ruminant species, a small transient rise in plasma progesterone, lasting four to nine days, precedes the first fertile estrous cycle. The detailed endocrine profiles of the estrous cycle in reindeer are generally in accordance with those found in sheep. Some research suggests that some females may experience two or more short cycles prior to the onset of full length cycles.[2,5]

A peculiarity in reindeer is the relatively short length of standing estrus when compared to that of other ruminants. Studies conducted at the University of Alaska revealed an average standing estrus time of one hour (range one to three hours).[3,4]

Females will continue to cycle well into spring (as late as April) having six to eight cycles through the winter. The transition into anestrus has been reported to occur with abrupt cessation of luteal activity or the formation of a persistent corpus luteum, which can persist into the next breeding season.[2]

As reported in other domestic species, in reindeer the introduction of a bull prior to the initiation of estrous cycles significantly hastens the onset of ovarian activity by two weeks and also results in synchronicity of calving the following spring.[3]

Estrous synchronization, super ovulation and artificial insemination

Growing interest in the truncation of the breeding season and artificial insemination have largely focused on synchronizing estrus.[7] Among captive reindeer in Alaska, two 15-mg IM injections of prostaglandin F2α (PGF2α; Lutalyse,® Pharmacia and Upjohn Company, Kalamazoo, MI) administered ten days apart resulted in leuteolysis and a single 15-mg injection at six weeks after conception terminated pregnancies.[2]

Attempts at artificial insemination (AI) in reindeer have been met with mixed results.[8] Most published reports give no information on either the methods employed or the results obtained. As with AI in other species, it is labor intensive and requires the ability to collect and store semen, as well as the ability to synchronize or recognize estrus in the female for appropriately timed insemination. Frozen semen AI successes have only been reported in a handful of cases.[9,10] The use of an ovine controlled drug release device (CIDR), has been described in both seven and 14 day protocols with timed artificial insemination occurring 44-60 hours following CIDR removal.[9,11]

In one study, an injection of cloprostenol (250 μg, im) administered at CIDR removal and a gonadotropin releasing hormone (GnRH) injection (100 mcg im) administered at artificial insemination (44 hours after CIDR removal) resulted in a 66% pregnancy rate.[9] Attempts at concurrent super ovulation with follicle-stimulating hormone (FSH) have resulted in a poor recovery rate of only 20%.[12] Another difficulty presented in transcervial artificial insemination is the reindeer cervix anatomically resembles that of the ewe, thus hindering the ability to readily pass the inseminating tube into the uterus.[8]

Pregnancy detection

Progesterone. Studies have been published on both Norwegian and Alaskan reindeer detailing the endocrinology of pregnancy. Progesterone concentrations show great variation during pregnancy (range of 2.4 - 14.28 ng/ml), both within and between animals, but do reliably increase immediately after conception to mean levels of 5.89 ± 0.09 ng/ml where it remains until parturition.[2,3,13,14] This is consistent with other species that are dependent on luteal progesterone production throughout pregnancy. Studies show that the reindeer placenta does produce progesterone; however, this contribution is not clearly evident in the progesterone profile.[15] As with other species, cyclic progesterone levels in non pregnant females can overlap those found in pregnant females and thus make peripheral progesterone an unreliable method of pregnancy detection.[2]

Pregnancy-specific protein B (PSPB). Pregnancy-specific protein B has been used to successfully detect pregnancy in populations of wild and domestic caribou and reindeer. Pregnancy-specific protein B appears in maternal plasma at 4.4 weeks (range 4-5 wks) after mating.[16,17] Blood samples should be collected six weeks following breeding. Reindeer owners in the continental United States, however, typically wait until after the seasonal reindeer activities and will draw blood for PSPB pregnancy determination in late December.

Trans-rectal ultrasonography. Given that reindeer are habituated to handling and restraint, trans-rectal ultrasonography is a useful modality for pregnancy detection. An advantage of ultrasonography is the application in the field and its ability to produce immediate results. It also allows for fetal measurements and assessment. It is routinely used between 35 and 60 days of gestation, although earlier detection is achievable. Beginning at week 20 of gestation and onwards it can be difficult to detect the fetus, since the gravid uterus is displaced ventrally and becomes unreachable for the ultrasound transducer.

Antler retention. Retention of antlers in pregnant reindeer cows has long been a technique of wildlife biologists to assess pregnancy status in wild caribou.[18] In reindeer it is not always a reliable predictor of pregnancy. Antler retention into mid-April can be used to infer pregnancy, although the contrary is not true; a portion of pregnant females often cast their antlers prior to calving.[2,19]

Gestation Length and Parturition

Reindeer have short and highly synchronized mating and calving seasons. Reported gestation length is highly variable. Published ranges exist from 198 to 240 days.[2,20] Variation may be due to environmental and latitude effects or different genetic stocks.

It has also been hypothesized that part of this variability is due to the limited reliability of observations of estrus and breeding, and therefore inaccurate estimates of conception date. Nevertheless, an estimated 90% of reindeer females are mated in a 10- to 21-day interval and give birth in an equally synchronized manner.[21] Several studies have documented a negative correlation between gestation length and conception date.[22] Although the underlying mechanisms responsible for this gestational plasticity and enhanced calving synchrony are not fully understood, it is assumed that the primary advantage of synchronized parturition is the fact that fewer neonates are lost to predation.[23]

Twinning is unusual in reindeer and caribou, with a plurality of twins not surviving birth.[24] Dystocia is somewhat uncommon in reindeer, but does occasionally happen. As with other cervids and small ruminants, manual correction of the malpresentation can be accomplished easily by a veterinarian. Malpresentations in reindeer are the same as those described in sheep and goats. Successful cesarian sections have been performed in a field setting using a lumbar approach similar to what is commonly used in sheep and goats. Muscular layers of the reindeer abdomen are much thinner than other cervids, and closure is difficult without a surgical assistant aiding in apposition and closure of the lateral abdomen.

Male reproductive management

Rut physiology

Antler development in the male occurs at the rapid rate of one to two inches of new growth per day. Antlers begin to develop in late winter and continue through the end of July.

The first sign of the pending rut is the cleaning of velvet from the antlers. Bulls will generally begin this process in late August. This process of antler cleaning is triggered by rising levels of testosterone and complete removal of velvet occurs rapidly, often within a 12-hour period. Intense aggressive displays follow with territorial marking and sparring similar to other cervid species. Rut behavior also includes a self marking display of hunching and urinating on the hind feet, termed trampling-urination, accompanied by distinctive vocalizations referred to as grunting or barking. The rut generally lasts through late November.

Voluntary food intake dramatically decreases at the onset of the rut. Males often will cease eating altogether at the height of rut. Reports suggest that it is common for males to lose up to 23% lean mass.[25] This body mass reduction occurred in all males over two years of age, regardless of social hierarchy. Careful management is required for all males during the rut. It is important to note that even the most docile and tractable bulls will become extremely aggressive and dangerous and cannot be trusted until the rut has ended.

Anesthetic unpredictability is reported in virtually all species of rutting males, but reindeer are particularly susceptible to the effects of cylohexamines (ketamine) and alpha-2 agonists (xylazine) during the rut. Deaths have been reported from a single 15 mg dose of xylazine.[2] Whenever possible, general anesthesia should be avoided in male reindeer during the breeding season.

Semen collection

The use of an artificial vagina and teaser cow have been described for semen collection in reindeer. The use of an electroejaculator has also proven to be a reliable method of collection using a standard ram probe. An unusually high rate of urospermia has anecdotally been reported following electroejaculation of male reindeer.

Reindeer semen is of poor quality, when compared with other domestic species. Reported progressive motility in freshly collected sample is often below 50% and abnormal spermatozoa approaches 50% on morphological examination.[26] These deformities seem to be generally equally distributed among primary and secondary abnormalities. Semen cryopreservation continues to be a challenge in reindeer, with most frozen-thawed samples having very poor motility.

Hormonal control of the rut

Medroxyprogesterone acetate (Depo-Provera,® Pharmacia and Upjohn Company, Kalamazoo, MI) has been used by reindeer owners for many years now to calm male reindeer and reduce aggression during rut.[2] Although it is not approved for use in reindeer, it is often administered in a set of two injections. These are administered in August and October through an intramuscular injection at a dose of 200-400 mg per animal. No specific studies have looked at the impact of this drug on spermatogenesis, fertility, future breeding or semen quality.

Antler development and casting in castrates

Unlike many other deer species, reindeer steers continue to grow massive antlers following castration. Steers occasionally have incomplete velvet removal and delayed antler shedding. Reindeer owners commonly use implants containing 10 mg of estradiol benzoate and 100 mg of progesterone (Synovex-C, Zoetis, Parsippany, NJ) placed at the base of the ear of castrates during October. Two pellets are inserted per animal. Steers will rub velvet and cast their antlers in a similar fashion to intact males following treatment. Subsequent antler cycles continue without interruption.

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Infertility of female and male camelids

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Introduction

Reproductive capacity is an important component of production and profitability within the camelid industry. Poor reproductive efficiency is a major problem and infertility represents the most common complaint in practice. Camelids have the lowest reproductive efficiency of all domestic farm animal species, where even the smallest decreases in fertility can have significant effects. Camelids present several anatomical and physiological reproductive differences (induced nature of ovulation, overlapping follicular waves, importance of corpus luteum [CL] for pregnancy maintenance, traumatic intrauterine copulatory mechanism, duration of mating, semen viscosity, etc.) compared to other large animal domestic species which may offer some challenges in the diagnosis and treatment of infertility.

The objectives of the present paper are to discuss the most commonly encountered female and male reproductive tract disorders resulting in infertility as seen in practice, along with the approach to diagnosis and treatment. Throughout the paper unless a specific species is mentioned, the term South American Camelids (SAC) will be used to refer to alpacas (*Vicugna pacos*) and llamas (*Lama glama*). The term camels will refer to all old-world camelids (*Camelus dromedarius* and *Camelus bactrianus*). The term camelids will be used to include all of these species.

Keywords: Camelid, infertility, ultrasonography, repeat breeding, endometritis, testicular degeneration, poor libido

Common presenting complaints

In female camelids the most common presenting complaints to veterinarians related to infertility can be categorized into repeat breeding, early pregnancy loss, abnormal behavior (continuous receptivity or rejection of the male), and visible abnormalities of the external genitalia.[1] Reproductive efficiency may be approached from a herd perspective or an individual perspective.[2] In a large number of breeding systems, mating is based on receptive behavior and not on specific ovarian follicular status. Most breeders use behavioral signs for pregnancy diagnosis (spitting and attempts to escape the male in SAC and tail curling in camels) at 14 days after mating.[1, 2] Arriving at a definitive diagnosis of the cause of infertility requires a thorough evaluation that should include a comprehensive history (thorough review of all breeding records and management practices), physical examination, and complete evaluation of the reproductive organs. An important consideration in the evaluation of the infertile female is that monitoring over at least one reproductive cycle (from follicular growth to mating to pregnancy diagnosis) may be necessary to determine a definitive diagnosis.[1-3] In some cases, a diagnosis may be made on a single examination.

Male camelids are often selected as herd sires based on pedigree, fiber quality, conformation, or performance, and not on standardized reproductive parameters.[4,5] Infertility in males is most often noticed when there is an inability to complete mating or when bred females do not become pregnant. As with evaluation of infertility in the female, determination of a definitive diagnosis in the male also requires a thorough evaluation including a comprehensive history, physical examination, and complete evaluation of the reproductive organs.[4, 5] In some cases, males may present for examination under emergency situations (sudden increase in testicular size, acute preputial swelling, paraphimosis etc.).

Breeding soundness examination of the female

Infertility workup in the female requires a systematic approach and timing of the initial examination is very important to gain maximum information. If a female does not become pregnant after a maximum of three matings (mating only once per week based on strong receptive behavior) she should be presented for a breeding soundness examination.[2] It is recommended that females are presented for an

evaluation 14 days following mating.[2] This examination should start with a complete health/reproductive history and physical examination. Physical examination should include overall body condition, examination of the major organ systems, and evaluation for clinical signs of infectious disease. Examination of the external genitalia should be emphasized. The vulva should be inspected for size, conformation, and presence of any abnormalities. Special attention should be given to evaluation for congenital defects in maiden females (discussed in more detail below).[1,2] A reproductive examination will consist of examination of the external genitalia (described above), transrectal ultrasonography, vaginal examination (digital examination and vaginoscopy), and in some cases hysteroscopy.[3,6] Additional diagnostics include endometrial cytology and culture, endometrial biopsy, endocrinology evaluation, laparoscopy/exploratory laparotomy, and cytogenetic evaluation.[3]

Transrectal ultrasonography is an essential part of the examination of the female camelid. Transrectal palpation alone is not accurate in assessing ovarian structures or evaluating the cervix and often times the size of the female may limit the ability for transrectal palpation to be performed. Follicular waves are overlapping[7] and ultrasonography allows for precise evaluation of follicular and luteal activity within the ovary. The dominant follicle grows (0.6 mm per day in SAC and 1.5 to 2 mm per day in camels) until it reaches a maximal size (nine to 14 mm in llamas, eight to 12 mm in alpacas, and 12 to 25 mm in camels).[2,7-9] The CL is easily visualized by day 4 after mating or induction of ovulation. Uterine tone and edema increase during the follicular phase (maximal in the presence of mature follicle). The uterus becomes relaxed during the luteal phase and pregnancy. Transrectal ultrasonography is performed using a 5 to 7.5 MHz linear transducer. For alpacas, the transducer is mounted on a handle to allow manipulation without inserting the hand into the rectum.

Digital examination of the vestibule-vaginal area should be performed on all maiden females (potential persistent hymen or vaginal segmental aplasia) and cases where vestibular or vaginal adhesions are suspected.[2] Vaginal and cervical examinations are performed using a sigmodoscope in alpacas and a tube vaginoscope in llamas and camels.[1,2] The cervix should be clearly visualized during normal examination. Hysteroscopy may be recommended for more thorough evaluation of the cervix and evaluation of the endometrium for presence of scarring or adhesions.

Endometrial cytology and culture should be a part of any infertility workup. An equine double-guarded brush (cytology) or swab (culture) is introduced through the cervix via a speculum (alpacas) or by recto-vaginal manipulation (llamas and camels).[1,2] Cultures should be performed for aerobic and anaerobic bacteria, *Ureaplasma*, *Mycoplasma*, and fungi.[1,2] Endometrial biopsy should be considered in cases presented for infertility, chronic endometritis, or a history of recurrent pregnancy loss/abortion. An equine endometrial biopsy punch can be used in llamas and camels. For alpacas, the use of a Turret rectal biopsy punch is recommended.[1,2,10] Targeted biopsies can be performed in combination with hysteroscopy. Although a grading system for histological evaluation has been proposed in camelids (Table 1),[11] it is not widely used in a clinical setting.[4,10] These techniques should be performed when the female has the presence of a dominant follicle and the cervix is open.

Table 1: Classification of endometrial biopsy and potential effect on fertility[2,10,11]

Biopsy Category	Histopathologic Characteristics	Effect on Fertility
Grade 1A	Normal endometrium	Normal conception rates
Grade 1B	Few lymphocytes within the endometrium. Siderophages present.	Low-grade infection or remnants of previous inflammation. Mild surface irritations may indicate reaction to breeding. May be postpartum or post-abortion (siderophages).
Grade 2A to 2B	Active and acute, chronic, or chronic active endometritis. Chronic inflammation tends to be more deeply located in the endometrium, compared with active and chronic active inflammation.	Interferes with conception and may cause early embryonic death.
Grade 3A	Chronic endometritis with glandular fibrosis.	Interferes with implantation and placentation. May cause early embryonic death.
Grade 3B	Uterine neoplasia	Pregnancy loss or abortion.

Breeding soundness examination of the male

Infertility workup in the male, as with in the female, requires a systematic approach. Breeding soundness examination should include complete health/reproductive history (detailed description of presenting complaint), general physical examination, and thorough examination of the reproductive organs/genital system. Historical information should include age, breeding records (number of females, breeding frequency, type of mating), previous health problems, and current reason for examination.[4] Poor conformation may impair the ability of the male to mount for mating and prolonged febrile conditions can affect spermatogenesis.[4] Physical examination should include overall body condition, examination of the major organ systems, and evaluation for clinical signs of infectious disease. Examination of the external genitalia should be emphasized. The scrotal skin is thin and smooth. It should be examined for evidence of bite wounds or other abnormalities. In older males the scrotum may appear more pendulous.[4] Testes should be present in the scrotum at birth in SAC. Testes enter the scrotum at two to three years of age in camels.[5] Examination of the prepuce may require restraint in lateral recumbency. Sedation or anesthesia may be required to exteriorize the penis.[4] Preputial attachment is normal in young, prepubertal males. In SAC the penis should be completely free in all males by the age of three years.[12] A reproductive examination will consist of examination of the external genitalia (described above), testicular measurements, testicular ultrasonography, evaluation of the accessory sex glands, evaluation of mating ability, and semen collection/evaluation. Additional diagnostics include testicular biopsy, endocrinology, trace mineral analysis, and cytogenetics.[4,13]

Examination of the testes and epididymides includes palpation, measurement, and ultrasonography. The testes should be smooth, firm, resilient to palpation, and nearly of equal size. Normally only the tail of the epididymis is palpable. Testicular size is an important indicator of sperm production ability and measurements should be taken with calipers or ultrasonography. Ultrasonography of the testes is important to detect problems that cannot be identified on palpation. Examination of the accessory sex glands (bulbourethral glands and prostate) may be recommended in some cases and is limited to ultrasonography.

Requesting owners to present a receptive female with the male for evaluation is imperative for assessment of mating ability. During this evaluation, the progression of a normal behavioral pattern should be recorded (along with duration) including vocalization, chasing, mounting, intromission, and duration of copulation.

Semen collection in camelids can be difficult due to the nature of copulatory behavior and process of ejaculation over an extended period of time. Techniques for collection of semen include use of an artificial vagina (in the case of trained males),[14] electro-ejaculation, or post-coital aspiration from the female.[4,5,12] In camels, the use of an artificial vagina is the preferred method for semen collection.[5] Semen collected by post-coital aspiration typically contains blood contamination from the female and may provide a very small sample. In the authors' practice semen evaluation was performed on samples collected by post-mating aspiration in the majority of alpaca cases (100% for routine breeding soundness examination and 59.2% for males presented for infertility).[15] Electroejaculation was only performed in males that presented with a complaint of infertility.[15] Electroejaculation requires general anesthesia with the male placed in lateral recumbency. An electroejaculator probe with linear, non-circumferential electrodes should be used. The electrodes should be placed at the level of the prostate (depth determined by transrectal ultrasonography). Stimulation should be done with extreme care starting with low voltage until erection is achieved. Urine contamination is a potential complication and males should be encouraged to void the bladder prior to anesthesia and electroejaculation. Studies have demonstrated that electroejaculation consistently resulted in ejaculate volumes of 0.25 to 1.75 mL in alpacas and did not produce an increased stress response from anesthesia alone.[16,17]

Examination of semen should include motility, volume, color, viscosity, concentration, and morphology. Camelid semen is very viscous (attributed to secretions of the bulbourethral glands)[12] and initial evaluation of motility will demonstrate oscillation prior to liquefaction of the sample. Progressive motility, as described in other species, is difficult to appreciate.[12] Individual motility can range from 20 to 95%.[18] Concentration is generally estimated using a hemocytometer.[12] Morphology assessment is

similar to other species by examination with stained smears (eosin/nigrosine and Diff-Quick). Preparation of morphology slides may be difficult due to the viscosity of the semen. Several studies have demonstrated highly variable normal morphology.[14,17] Normal males should have at least 50% morphologically normal spermatozoa.[18] Normal cytology should not reveal erythrocytes or leukocytes in ejaculates collected by artificial vagina or electroejaculation.[4] Research is needed to determine suggested parameters of an ejaculate that would be required for maximal fertility.[4]

Testicular biopsy is not a routine part of the breeding soundness examination in the male. In some cases however, a diagnosis and prognosis of the male's fertility cannot be reached based on physical examination, ultrasonography, and semen evaluation. Testicular biopsy can help distinguish azoospermia of testicular or non-testicular origin.[4] Several techniques have been described including fine needle aspirate, large bore needle (14 gauge) core biopsy, wedge biopsy, and split needle biopsy. The recommended technique for collecting testicular biopsy samples is to use a spring-loaded split-needle biopsy instrument.[18]

Assessment of female infertility

Infertility in females can be broadly categorized into congenital and acquired abnormalities. Several congenital abnormalities of the reproductive tract have been described in SAC. The most common congenital disorders that might be seen in practice include ovarian dysgenesis/hypoplasia, segmental aplasia of the uterus, vaginal aplasia, persistent hymen, and vulvar atresia.[1] Females with ovarian hypoplasia will present as maidens, females with a history of multiple matings/continuous receptivity, or persistent rejection of the male. They tend to be taller have more fine fiber than normal females.[2] Transrectal ultrasonography over several days will demonstrate a small uterus and either small follicles that do not progress to a dominant size or inability to visualize the ovaries entirely.[1] Cytogenetics may reveal chromosomal abnormalities such as 73XO, 75XXX, 74XX/74XY, minute chromosome).[2 19,20] The condition is confirmed by laparoscopy. Segmental aplasia of the reproductive tract may occur at the level of the uterine tube (formation of hydrosalpinx) or at the level of the uterine horn (uterus unicornis). These females will often present with normal cycles and ovulation but fail to achieve or maintain pregnancy. Diagnosis of uterus unicornis may be achieved by ultrasonography and confirmed by hysteroscopy or laparoscopy. Females with vaginal aplasia, persistent hymen, or vulvar atresia may present with a history of rejecting the male, persistent straining or pain during mating, or have excessive vulvar swelling after mating.[1] Transrectal ultrasonography generally demonstrates accumulation of fluid or mucus in the vagina/uterus (hydrometra, mucometra). Confirmation is achieved by examination of the external genitalia and vaginal examination. Digital palpation is often sufficient to determine an occlusion at the level of the vestibulum. There is no treatment for complete vaginal aplasia. Females should be separated from males because they will continue to be receptive and breeding may lead to complications. An imperforate hymen is corrected by making an incision through the tissue with a scalpel, however potential genetic correlation and future breeding should be discussed with the owner. Incomplete perforation of the hymen (vestibular narrowing) is fairly common and may be managed successfully with bougienage.[1] Vulvar aplasia is also relatively common and surgical repair is controversial.

The most common behavioral complaint in females is rejection of the male in absence of pregnancy.[2] Spontaneous ovulation does occur and is more common during the postpartum period. Aggressive behavior and infertility may be associated with ovarian tumors (granulosa theca cell tumors) and unilateral ovariectomy may re-establish ovarian function of the contralateral ovary in some cases.[1,21] Failure of ovulation is a common problem in camelids. Anovulatory follicles can become hemorrhagic and in some cases luteinize and produce progesterone resulting in rejection of the male.[2,22] It is important to note however that anovulatory follicles do not necessarily inhibit the growth of other follicles. Treatment with human chorionic gonadotropin (hCG) or gonadotropin releasing hormone (GnRH) may be attempted. Ovulation failure may also be observed in females with vagina/cervix abnormalities that may inhibit the normal mechanism of induction of ovulation.[2] Ovarian follicular activity may be reduced in females that are lactating, obese, are heavily parasitized, are advanced in age, or have experienced severe

systemic disease.[2] Acquired ovarian inactivity is commonly observed in lactating camels.[2,5]

In SAC and camels the major owner complaints on presentation to a veterinarian were "repeat breeding" and "early pregnancy loss" (76 and 18% respectively) in one study.[3] Repeat breeding in a female with a history of at least one normal pregnancy/birth may be due to inappropriate breeding management, endometritis, failure of ovulation, failure of fertilization, or early embryonic loss.[2] Management of reproduction in camelids can be complicated because female receptivity does not correlate to mature follicular size but rather absence of luteal tissue (progesterone).[2] Failure of fertilization may also be due to uterine tube or ovarian bursal abnormalities including inflammation, pyosalpinx, or hydrosalpinx.[2] In a recent study involving dromedary females, clinical endometritis, ovarian hydrobursitis, and vaginal adhesions were the most common clinical findings in females examined for repeat breeding with regular heat intervals, refused matings, or repeat breeding with long heat intervals.[23] Ovarian hydrobursitis is a long-standing reproductive syndrome in dromedary camels characterized by fluid accumulation and encapsulation of the ovary.[24] In camels, ovario-bursal adhesions and ovariobursitis have also been reported. In some cases these conditions may be associated with *Chlamydophila* infections.[24-26] Bilateral and unilateral left and right ovarian hydrobursitis was observed at frequencies of 42%, 46%, and 12%, respectively.[23]

The major contributing factors to endometritis are over-breeding, unaddressed postpartum complications, and improper obstetrical manipulations.[23] Endometritis can be classified as acute, sub-acute, or chronic. Nutritional deficiencies such as selenium and copper may also be linked to an increased incidence of endometritis. In cases of endometritis females will likely present for regular return to cyclicity and mucopurulent discharge.[22] Transrectal ultrasonography may demonstrate areas of increased echogenicity and thickening of the uterine wall.[2,22]

Cytology and culture are necessary in cases of suspected endometritis. Endometiral biopsy should also be considered in the diagnosis of endometritis and can be a prognostic indicator of future fertility.[27] Bacteriological culture should include sensitivity testing for the major antibiotics. The most common bacteria isolated from cases of camelid endometritis are *Escherichia coli*, *Streptococcus equi zooepidemicus*, *β-hemolytic Streptococci*, *Enterococcus spp*., coagulase negative *Staphyloccocus* spp., *Proteus spp*, *Enterobacter aerogenes*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Trueperella pyogenes*.[2,23,28] Venereal transmission should be considered in case of herd infertility or abortion outbreaks.[2,29] *Pseudomonas aeruginosa*, *Campylobacter fetus fetus*, *Tritrichomonas foetus*, *Aspergillus* spp., and *Mucor* spp. have been isolated from infertile camels.[2,28,30]

Therapeutic management of endometritis involves uterine flushing, intrauterine antibiotic infusion, systemic antibiotic administration, or a combination.[1,2] Uterine flushing is accomplished with lactated Ringer's solution (LRS) or a proprietary equine uterine lavage solution. Administration of oxytocin may improve uterine clearance. Infusion with 30% DMSO solution or N-acetylcysteine prior to uterine lavage may be considered in chronic cases with thick mucopurulent discharge.[2] Biofilm formation is a characteristic of many organisms found in endometritis and the addition of buffered chelating agents (tris-EDTA and Tricide®) may help dissolve the biofilm and improve antimicrobial action.[2] A 4% metacresol-sulfonic acid and formaldehyde solution (Lotagen) has provided good results for treatment of endometritis and metritis in camels.[29] Most antibiotics which can be used *in utero* can also or alternatively be used systemically and achieve very good concentration in uterine tissue. Success of treatment of endometritis depends on the duration of infection and females should be re-examined after a period of sexual rest.[2] Chronic endometritis will lead to development of degenerative changes or fibrosis. In these cases the best option in valuable females is embryo transfer.

Early pregnancy loss is a common complaint in camelids as mentioned previously.[2,3] Pregnancy loss may be the result of cervical incompetency (sequela to dystocia/obstetrical manipulation), uterine abnormalities (scarring, fibrosis, adhesions, etc.), genetic abnormalities, systemic disease (trypanosomiasis in camels), twinning, or luteal insufficiency.[2,28] Most early embryonic loss occurs before 45 to 60 days of gestation.[1,2] Management of recurrent pregnancy loss is usually managed with advanced reproductive techniques such as embryo transfer.[2] Uterine fibrosis should be considered in

older females or those with long-standing infertility or pregnancy loss.[22] Endometrial biopsy is diagnostic and provides prognosis for future fertility.[22]

Other causes of infertility include vaginal or cervical adhesions (may lead to development of pyometra or mucometra), uterine lacerations, ovarian neoplasia, uterine neoplasia, and peri-uterine adhesions. Referral is sometimes warranted for diagnostics such as hysteroscopy, laparoscopy, or fluoroscopy.[2,3] A high incidence of pyometra was observed in middle-aged female dromedaries presented for infertility.[27]

Assessment of male infertility

Infertility in males is most often noticed when there is an inability to complete mating or when bred females do not become pregnant.[18] Inability to complete mating could be due to poor libido, abnormal mounting, erection failure, or ejaculation failure. Mating includes several steps that need to be observed for any abnormalities. Poor libido could be behavioral or functional. Young males may not have been socialized appropriately in groups (show animals), are inexperienced/timid, or have been reprimanded by handlers.[4,18] It becomes important with young males to introduce them slowly to breeding. In adult proven males, deterioration of libido can be due to systemic disease, musculoskeletal abnormalities, or heat stress. Endocrinology testing can determine if poor libido is due to low testosterone.[4] Males with musculoskeletal disorders may complete mating if they are supported during mating. Small males may not be able to maintain a normal mating position and achieve intromission.[18] In camels a seasonal effect (heat stress) is present and poor libido (with corresponding decrease in testosterone) is observed during the summer months.[5] Poor libido can also be the result of overuse. Excessive work load may result in a lack of libido. Dromedary bulls that are overused within the breeding season, and especially out of the breeding season, may demonstrate a decrease in libido, be slower to breed, and in some cases may fail to ejaculate.[5] In such cases it is recommended to remove the male from work and provide a period of rest.[5] Testosterone therapy in dromedary bulls increased libido but resulted in a significant reduction in spermatogenesis and sperm concentrations, and is therefore not recommended.[5]

Semen is deposited deep into the uterine horns during copulation in camelids.[12] Erection failure in young males may be due to immaturity (lack of preputial detachment). On evaluation, inability to exteriorize the penis or erection failure could be due to congenital abnormalities or acquired conditions. Congenital abnormalities that have been identified include persistent frenulum, short penis, or abnormal function of the penile retractor muscle.[4,18] Acquired conditions such as preputial stenosis or adhesion formation may be due to severe inflammation from trauma (breeding the ground, fighting injuries, preputial prolapse, paraphimosis, etc.). Phimosis in camels may result from ischemic necrosis of the penis following application of straps for movement in and out of trucks for transport.[5] Prognosis for any of these types of injuries is guarded to poor. Failure of ejaculation may be due to painful conditions (musculoskeletal abnormalities, hair rings, penile warts, abnormal vaginal conformation of the female) that force the male to interrupt breeding.[4,18]

Lack of achieved pregnancies may be the result of abnormalities leading to derangements in ejaculation (absence of ejaculation (aspermia) or incomplete ejaculation (oligospermia)) or within the spermiogram (azoospermia, oligozoospermia, teratoozospermia). Measurement of seminal plasma alkaline phosphatase is not a marker of ejaculation in alpacas.[17] Testicular abnormalities are often identified during routine breeding soundness examination, which is why any male that is considered as a herd sire should be evaluated early to provide a baseline and rule out potential congenital abnormalities. The most common congenital abnormalities found on routine evaluation are testicular hypoplasia, cryptorchidism/ectopic testes, and testicular/epididymal cysts (Table 2). Some chromosomal abnormalities may result in teratozoospermia and cytogenetics may be necessary for diagnosis.

Table 2: Documented diseases of the reproductive organs in the male SAC at the WSU-VTH Theriogenology service[18]

Prepuce	Penis	Testis and epididymis	Accessory sex glands
Preputial edema (heat stress)	Prolapse	Cryptorchidism	Prostate hypertrophy
Obstruction	Paraphimosis	Ectopic testis	Prostate abscess
Laceration	Inflammation	Hydrocele	
Prolapse	Ulcerations/Abrasions	Testicular degeneration	
Necrosis	Hair ring	Testicular hypoplasia	
Inflammation (Posthitis)	Penile warts	Testicular cyst	
Warts	Urethral rupture	Orchitis	
Phimosis	Urethritis	Epididymitis	
	Urolithiasis	Epididymal segmental aplasia	

Testicular hypoplasia, ectopic testes, and bilateral cryptorchidism often result in severely impaired testicular function (azoospermia) and complete sterility in some cases.[5,18] Congenital segmental aplasia along the epididymal ducts or ductus deferens may also result in azoospermia.[4] In the case of unilateral cryptorchidism males may be fertile but should be removed from the breeding pool.[31] Rete testis and epididymal cysts are not uncommon, however they are not palpable and must be diagnosed with ultrasonography.[32] They should be documented and monitored.[4,18] Their role in infertility is variable.[15] Ultrasonographic examination performed on 173 male alpacas presented for castration identified rete testis cysts in 18.5% of males.[33] Following castration disruption of spermatogenesis was evident in testes with large cysts and examination of the epididymis of affected testes demonstrated 20% were completely void of spermatozoa.[33]

Testicular hypoplasia has to be differentiated from testicular atrophy following injury to the testis, testicular disease/degeneration, heat stress, or debilitating disease.[18] Testicular degeneration is not uncommon in older breeding males or those that have experienced severe systemic disease or heat stress.[18] Testicular degeneration is probably the most common cause of infertility due to testicular pathology. The testes are small and either soft or hard/fibrous on palpation.[18] In these cases evidence of oligozoospermia and teratozoospermia or azoospermia may be observed. In camels anabolic steroids are sometimes used in attempt to improve weight gain, muscle growth, and performance in stud camels, resulting in reduced spermatogenesis and testicular size.[5] These drugs will have immediate effect, but may also have long-term effect.[5] Testicular degeneration is irreversible, however if azoospermia has not yet developed, it may be possible to mate the male on a restricted breeding schedule to achieve pregnancies.[4] Testicular tumors are rare. Testicular biopsy becomes important in providing a histological diagnosis of testicular abnormalities and is considered the gold standard for diagnostic evaluation of any case with azoospermia.[4] In cases of acquired abnormalities, 60 days of sexual rest may be recommended followed by re-evaluation.[4]

Scrotal trauma due to fighting with other males is common in camelids. Prognosis for reproductive function depends on the extent of the injury. Deep lacerations are often complicated due to testicular hemorrhage or development of infection. In some cases unilateral castration is recommended, if a single testicle is affected, to preserve fertility. Hydrocele can be due to inflammatory or non-inflammatory processes. The scrotum will become enlarged and initial diagnosis is based on palpation (generally non-painful). Confirmation is achieved with ultrasonography and visualization of fluid within the scrotal sac. Moderate hydrocele can be observed in males that are not well managed during summer months and will progressively decrease as the ambient temperature drops. Enlarged, painful testes on palpation could be the result of orchitis. Orchitis can result from trauma to the scrotum due to bite wounds from fighting.[4,5] The most common systemic diseases that may result in orchitis in camel raising areas include trypanosomiasis (*Trypanosoma evansi*) and brucellosis (*B. abortus, B. melitensis*).[5,28] Systemic antibiotics may be considered for treatment of infectious orchitis but in most cases are not efficacious and castration is recommended.

Conclusion

A diagnosis of infertility in the female can often be achieved with a methodical examination approach, although in some cases a diagnosis may remain difficult to attain. Evaluation of breeding records and discussion of appropriate breeding management protocols is vital. A breeding soundness examination is paramount to achieving maximal reproductive efficiency. Females should be mated once per week based on strong receptive behavior and if a female continues to be receptive after a maximum of three matings to a proven male, a breeding soundness examination should be performed.[2] In some instances of acquired subfertility or infertility a minimum contamination breeding technique could be implemented. This requires ultrasonographic monitoring of ovarian activity and breeding only when a mature follicle is present. In cases where females have experienced previous uterine infections broad spectrum antibiotics may be administered daily starting one day prior and continuing for three days following mating. Only males with high fertility should be used and copulation should be limited to a maximum of 15 minutes, following which ovulation should be induced with administration of hCG or GnRH.

Infertility in the male can have a significant negative impact within a production system. Reproductive disorders in the male generally carry a poor prognosis when associated with severe lesions or a decline in semen quality. Hemicastration may be recommended in some cases and should not affect the fertility of the male if the remaining testis is still normal.[4,18] Clients should be educated to examine the external genitalia frequently on prospective and current herd sires, and annual male breeding soundness examinations should be a routine part of breeding management as a screening tool for potential problems.[18]

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Embryo transfer in camelids

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Introduction

Camelidae (or camelids) are economically important in South America, Africa, and the Middle East. However, they have also become increasingly popular in other parts of the world including the United States. They are one of the only production animal species that can survive under some of the harshest climatic conditions on the planet. Reproductive capacity is an important component of production and profitability within the camelid industry. Embryo transfer (ET) and associated techniques, particularly multiple ovulations and embryo transfer (MOET), can provide clinical advantages such as improved genetics, reduction of generational intervals, and prolongation of the reproductive life of genetically superior females that are unable to carry a pregnancy to term.[1,2] Embryo transfer also provides a tool for research in areas including mechanisms of fertilization, embryo development, and maternal recognition of pregnancy where knowledge is lacking compared to other domestic species.

Successful non-surgical ET in camelids with birth of a live offspring was not reported in the llama until the 1980s.[3] Large-scale commercial ET became widespread first in the dromedary camel and later in the alpaca industry.[4,5] The objectives of the present paper are to discuss principles of reproductive physiology in camelids that are important for ET, to discuss donor and recipient management, to describe the non-surgical ET technique, and to discuss factors affecting the success of an ET program. Many of these aspects have been the subject of recent reviews.[4-6] Throughout the paper unless a specific species is mentioned, the term South American Camelids (SAC) will be used to refer to alpacas (*Vicugna pacos*) and llamas (*Lama glama*). The term camels will refer to all old-world camelids (*Camelus dromedarius* and *Camelus bactrianus*). The term camelids will be used to include all of these species.

Keywords: Camelid, embryo transfer, superstimulation, pregnancy

Principles of reproductive physiology in camelids

Reproductive physiology in the female camelid presents striking differences compared to other large animal domestic species.[7] Development of a successful ET program requires a thorough understanding of the physiological mechanisms controlling follicular dynamics, ovulation, fertilization, early embryo development, and maternal recognition of pregnancy (MRP).

Follicular dynamics in most of the camelid species have been defined through several studies.[8-10] Field and experimental observations have demonstrated that ovarian activity in the female camelid is not seasonal under optimal nutritional conditions. Follicular waves occur in an overlapping manner. The duration of follicular waves is variable (Table 1).[11]

Table 1: Reproductive parameters in female camelids[11]

Parameter	*C. dromedarius*	*C. bactrianus*	*V.pacos*	*L. glama*
Follicular wave phases duration				
Growth (days)	10.5±0.5	10.9 ± 3	3-9	3-9
Maturation (days)	7.6 ± 0.8	7 ± 4.2	2-8	2-8
Regression (days)	11.9±0.8	11.9 ± 4.2	3-8	3-8
Ovulatory follicle characteristic				
Minimum size (mm)	9	9	6	8
Growth rate (mm/day)	1.8	1.8	0.43	0.5-0.9
Average size (mm)	10-18	10-18	8-10	9-12
Maximum size (mm)	25	22	12	13
Incidence of anovulatory follicles (%)	40-50	?	5	10-40
Anovulatory follicle regression (days)	8-45	?	?	4-22
Corpus luteum characteristics				
Interval from mating to ovulation (hours)	32 to 40	30 to 48	28 to 30	27-36
Size (mm)	15-25	15-25	11-15	11-18
Day at CL maximum size	7.2±1.7	7.3	7-8	8
Day of luteolysis	10 ± 1.2	10.5	10-12	10-12

*Extreme variation in onset of postpartum ovarian follicular activity is primarily due to nutritional condition and effect on lactational anestrus and seasonality.

All camelids are induced ovulators, with ovulation occurring equally from both the right and left ovary.[7,12-14] The presence of an ovulation-inducing factor (OIF) within the seminal plasma was identified in recent studies in llamas and alpacas as β nerve growth factor (βNGF).[15,16] Both βNGF and endometrial inflammation (resulting from mating) are required to maximize the ovulation rate. Ovulation occurs on average 28 to 32 hours after mating. Double ovulations are not uncommon in most domestic camelids in good health and nutritional status.[13] Triple and quadruple ovulations have also been documented in the dromedary camel.[17] In situations where mating does not occur or ovulation is not induced, some follicular waves may lead to the development of large anovulatory follicles that could become hemorrhagic or luteinized. Anovulatory hemorrhagic follicles (AHF) are more frequent in camels and llamas and seem to be individual dependent.[11] The pathophysiology of the development of AHF is not well understood.[8]

During mating, semen is deposited deep into the uterine horns. A sperm reservoir is formed in the isthmus region of the uterine tube following mating.[18] Fertilization rates are high in alpacas, often exceeding 80% in well managed breeding programs.[8,13] Fertilization rates per mating are reportedly lower (50 to 75%) when females are mated based on receptivity versus mating based on the presence of a mature follicle.[8]

The camelid embryo reaches the uterus between 6 and 6.5 days after ovulation and fertilization (day 7.5 after mating). While fertilization can occur on either side, embryos that descend into the right uterine horn will migrate to the left uterine horn for attachment.[19] In alpacas, 83.3% of the embryos resulting from right ovarian ovulation were found in the left uterine horn by day 9.[19] Camelids are unique in the fact that greater than 98% of pregnancies are carried in the left uterine horn, suggesting that embryo migration to the left uterine horn is important to prevent luteolysis.[12,14,20] This is attributed to a difference in prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) release between the two uterine horns. Prostaglandin $F_{2\alpha}$ release from the right uterine horn is local, whereas its release from the left uterine horn is systemic.[12] Gross examination of the uterus from fetal, pre-pubertal, and non-pregnant female camelids has demonstrated that the left uterine horn is consistently larger when compared to the right uterine horn.[7,21]

Collection of camelid embryos from the uterus yields mostly hatched blastocysts.[8,19] The hatched blastocyst expands rapidly and starts to elongate on day 9. The mechanism of MRP remains poorly understood in camelids. Studies suggest that MRP has to take place relatively early after mating (day 8 to day 10) in order to prevent luteolysis. The corpus luteum (CL) is necessary for maintenance of pregnancy

throughout gestation. The size of the CL varies between 11 to 20 mm in SAC and 15 to 25 mm in camels.[8] A cavitary CL is considered normal. It is important to note that MRP occurs at a time when the embryo undergoes a rapid elongation as is observed in ruminants, however identification of a substance such as interferon tau has not been successful. By day 15 after ovulation attachment begins as the trophoblast becomes in contact with the entire endometrial surface.[8,22] A well-formed microvillus junction between the fetal and maternal tissues is established by day 45 in alpacas. Placentation in camelids is epitheliochorial, microcotyledonary, and diffuse.[8]

A major difference between SAC and camels is the timing of postpartum resumption of ovarian activity. While SAC can resume ovarian activity within ten days postpartum, camels have a long lactational anestrus, which is one of the reasons MOET can have a huge impact on the generation interval and genetic improvement in camels.[2,8]

Donor management

Donors should be selected based on the contribution of highly desirable genetic traits without the presence of genetic disorders. Donors should be healthy systemically with good conformation and in general should be reproductively sound to achieve maximum efficiency. Donor management can be the result of embryo collection on a per cycle basis without manipulation of the number of follicles or through the use of MOET.

Donor management without ovarian stimulation

Several considerations need to be taken into account before deciding on donor management with or without ovarian superstimulation. Collection of embryos without ovarian superstimulation may offer several practical advantages such as eliminating the need for a large number of synchronized recipients and facilitating easier manipulation of the reproductive tract for non-surgical collection, specifically in alpacas. In addition, embryo recovery results are more reliable.[5]

Donor females are mated when the follicle has reached a mature size and the uterus presents maximum tone and edema. Embryo collection is scheduled eight days after mating (approximately seven days after ovulation).[4-6] Studies on dromedaries using this system demonstrated a collection rate ranging from 60 to 100%.[11] In alpacas the embryo recover rate varies between 58 to 100%.[5,13,19,23] It is possible to collect up to two embryos per donor without stimulation. In a recent study on alpacas, the incidence of double ovulation and presence of two embryos in the uterus was 30% and 20%, respectively.[13] In the dromedary camel, collection every two weeks from the same female throughout the breeding season did not result in any adverse effect on fertility. The number of collections per season was 10±4.2 resulting in an average number of transferrable embryos per season of 8.5±3.1 and a mean number of pregnancies per donor per season of 4.1±1.2.[4]

Donor management with ovarian superstimulation

Commercial alpaca embryo transfer in Australia reports an average of 2.5 to 3 embryos per uterine flush with the potential for production of up to 21 embryos per individual per year using MOET.[5] Protocols for ovarian superstimulation in camelids have largely been modeled after ruminants. Follicle stimulating hormone (FSH) and equine chorionic gonadotropin (eCG) have been used primarily, either alone or in combination. Response to these hormones depends on timing of initiation of treatment in relationship to the follicular wave, dose and schedule of administration, and individual variation. Best results with FSH and eCG treatments are obtained when treatment is initiated in the absence of any follicles greater than 2 mm.[8] Elimination of dominant follicles prior to gonadotropin treatment includes manual ablation, ultrasound-guided aspiration of the dominant follicles, or initiation of treatment following a period of progesterone (with or without estradiol) treatment.[24,25] Progesterone treatments include daily progesterone injections, intravaginal devices (PRID, CIDR, medroxyprogesterone acetate sponges), or subcutaneous implants of norgestomet.[25,26] The length of treatment varies from seven to 14 days, however it is important to recognize that progesterone alone does not always control follicular wave emergence.[8] The combination of estradiol and progesterone in control of follicular waves has shown

variable results.[25,27] Initiation of gonadotropin treatment at a specific time (two to four days) following induction of ovulation has been shown to be produce more reliable results.[28]

Both ovine (oFSH) and porcine (pFSH) FSH have been used in stimulation of follicular development in camelids.[8] Detailed descriptions of FSH administration are often not clear in many publications. In the dromedary, oFSH was given twice daily for three to five days following a ten to 15 day course of progesterone treatment (100 mg per day).[24] Porcine FSH given twice daily in decreasing doses over three, five, or seven days after a ten to 15 day progesterone treatment also resulted in ovarian superstimulation of dromedary females.[4,24] The interval from pFSH treatment to development of mature follicles (10 to 16 mm in diameter) varies between six and eight days.[8] The number of embryos collected following superstimulation with FSH is variable (average of eight embryos).[4] The best superstimulation and embryo collection results in llamas were obtained following administration of pFSH twice daily for five days in decreasing doses for a total of 220 mg, however the embryo recovery rate in relationship to the number of CL was only 48%.[29] Alpacas reportedly produce a more variable response to superstimulation protocols than llamas.[25]

Ovarian superstimulation with eCG has been used in camelids following progesterone priming. In general, a single dose of eCG is administered intramuscularly (IM) one day before or on the day of completion of a five to 15 day progesterone regime. The dose of eCG varies from 1500 to 6000 IU in camels, 500 to 2000 IU in llamas, and 500 to 750 IU in alpacas. When eCG is administered to female dromedaries with no dominant follicle, the interval from treatment to mating is relatively constant (eight days), however follicular response (zero to 19 follicles) is variable with a portion of females not responding.[8] In llamas, eCG (1000 IU) following progesterone priming, resulted in variable follicular response (zero to 13 follicles), variable ovulations (0 to 7), and variable embryo collection (zero to six embryos).[29] The main disadvantage of eCG use is the high incidence of follicular luteinization and disturbance of ovulation (anovulatory follicles), potentially due to its long half-life. There is also a potential for females to become refractory to eCG following multiple administrations. The use of eCG in combination with FSH has been attempted in SAC[25] and camels.[8]

Superstimulation protocols are far from perfect and ovulation response and embryo collection are highly variable. Some donors may become refractory to hormonal stimulation, whereas others may be overstimulated. Sources of variation to be investigated in response to superstimulation include species, individual animal variation, and breed variation.

Mating management

The best mating management is based on ultrasonographic monitoring of follicular size. Transrectal ultrasonography is ideal for monitoring follicular development and uterine tone/edema. Mating should be performed when follicular size is between 12 and 16 mm in diameter in camels, eight to ten mm in diameter in llamas, and seven to ten mm in diameter in alpacas. While mating is sufficient for induction of ovulation, in most situations human chorionic gonadotropin (hCG; 1500 to 3000 IU for camels, 750 to 1000 IU for SAC) or gonadotropin-releasing hormone (GnRH; 100 μg in camels, 50 μg in SAC) is administered immediately after mating to have a predicable ovulation response. While some authors have suggested two matings at 12 to 24 hour interval,[24] a single mating is likely sufficient unless the first mating was less than three minutes.[4,8] Ovulation of the donor is detected by transrectal ultrasonography or serum progesterone concentrations. Plasma progesterone concentrations reach high levels (>2 ng/mL) by day five following ovulation. In large scale embryo transfer operators, donors are examined by transrectal ultrasonography one to two days before embryo collection to estimate the number of CL.[4]

Embryo collection and evaluation

In practice, non-surgical collection of embryos is the standard. For the best recovery rates it is recommended to wait until 7.5 to eight days after mating for embryo collection. Supplies needed for non-surgical collection of embryos are similar to those used in bovine embryo collection.[30] In camels, the rectovaginal technique of catheterization of the cervix is performed in the standing position. The same

technique can be used in SAC, however alpacas and small llamas require sedation and sometimes caudal epidural anesthesia. Sedation may be achieved using butorphanol tartrate (0.05-0.1 mg/kg IM) alone or in combination with xylazine (0.05-0.1 mg/kg IM).[30] In alpacas the uterus is flushed with the female sitting in a sternal position.[8] Catheterization of the cervix in alpacas may be achieved with the aid of a small vaginoscope or a sigmoidoscope. Foley catheters (18 to 22 Fr gauge for camels, 12 to 16 Fr gauge for llamas, 5 to 10 Fr gauge for alpacas) are used for flushing the uterus.[4, 6]

The uterine horns are flushed separately or both at the same time using Dulbecco's phosphate-buffered saline (DPBS) or a commercial complete embryo flushing medium (Vigro® complete flush, Vetoquinol, USA) in small quantities (20 to 60 mL in SAC and 60 to 200 mL in camels).[11] It is important to allow the fluid to remain in the uterus for 30 to 60 seconds before recovering it.[30] The uterus is flushed with a total volume of 500 mL in SAC and 1 L in camels. The flushing medium is recuperated into an embryo filter.[4,6] Embryos recovered from the uterus in camelids are at the hatched blastocyst stage. Embryo recovery rates are higher in non-stimulated females when compared to superstimulated females as mentioned earlier. Donors are generally given a luteolytic dose of cloprostenol (250 µg IM) after flushing to prevent pregnancy in case of failure of embryo recovery.

Embryo searching may be conducted within the embryo filter or following transfer of the collection medium into a separate searching dish. The clinic and laboratory in which embryo collection and searching is performed should be maintained at a temperature between 20°C and 25°C and the area should be clean and free of dust.[11,30] Ideally the person handling the embryo should wear a cap and mask during the searching and evaluation process.[30] Embryo evaluation aims to determine the stage of development, size, and quality of the recovered embryos. The evaluation system used by the authors' classifies embryos into five grades according to their morphological characteristics and stage of development (Table 2).[11] Embryo grade affects pregnancy rate and determines suitability for potential cryopreservation.[11] In most fresh ET programs all embryos grade I to grade IV are transferred as long as they are hatched blastocysts.[30] Embryos should be washed and maintained in a holding medium prior to transfer.[11]

Table 2: Classification of uterine stage of camelid embryos

Grade	Characteristics
I	Excellent quality: Hatched blastocyst. Should be almost perfectly spherical between day 7 and day 8
II	Good quality: Some irregularities with very few extruded cells
III	Medium quality: Small with dark patches, irregularly shaped, extruded cells
IV	Poor quality: collapsed blastocysts, irregular contour with dark areas, light areas or tears
V	Non-transferrable embryos: collapsed very dark blastocysts, all stages of embryonic development still with zona pellucida (non-hatched)

Recipient management and embryo transfer

The ideal recipient is healthy and has successfully carried at least one pregnancy. Maiden and aged females should not be used where possible. Recipients should be screened for contagious diseases.[4] Nutrition and good body condition are important. Trace mineral supplementation should be provided as needed. In SAC pregnancy rates following ET were significantly higher in non-lactating recipient alpacas than lactating recipient alpacas.[31] Where possible all females should undergo a complete breeding soundness examination and females with an unknown reproductive history should be submitted for an endometrial culture and biopsy before being accepted into an ET program.[4]

Synchronization between the recipient and donor is extremely important for success after transfer of the embryo. The best pregnancy rates are obtained when the recipient has ovulated one to two days after the donor.[4,5,24,32] There are no reliable methods for synchronization of the donor and recipient ovulation however, and the availability of recipients becomes an important limiting factor for development of ET programs. Synchronization of follicular development in recipients with eCG when superstimulation is started in the donor is possible, however pregnancy rates after ET are usually low if

the number of CL is greater than six or lower than two in the recipient.[24] Pregnancy length after transfer varies from 349 to 420 days in dromedaries[4] and from 319 to 387 days in alpacas.[5]

All commercial embryo transfers are performed using a direct non-surgical technique. Embryos are loaded in 0.25 mL or 0.5 mL straws and transferred using a standard bovine ET gun.[4,5]

Factors affecting embryo transfer results

Success of an embryo transfer program is multi-factorial. Major factors that significantly affect success rate include day of collection, embryo quality, recipient age, and recipient body condition score.[5] The effect of season (winter and spring producing less embryos),[5] lactation status of recipients,[31] operator experience, and stress during the embryo transfer should also be taken into consideration. The effect of the side of transfer of the embryo in relationship to the CL likely results in increased pregnancy rates.[4] While it was not discussed in the current paper, factors such as artificial insemination with frozen-thawed semen, cryopreservation of embryos, and use of in vitro produced embryos in an ET program need to be considered also.

Overall success of a program is based on the percentage of offspring born or weaned. In a large commercial embryo transfer operation in alpacas the overall pregnancy rate and birthing rate were 43.2% and 41.9% respectively.[5] In dromedaries the embryo recovery rate and pregnancy rates in non-stimulated females were 94.6% and 42% respectively with the overall weaning rate for pregnant females between 70 to 100%.[4]

Conclusion

Embryo transfer programs have the potential for improving genetics and providing a means for further reproductive capabilities in females experiencing subfertility/infertility. While embryo transfer programs are commercially available, the overall efficiency of these programs is still in need of evaluation. More studies are needed to evaluate sources of variation in the superstimulation response. Examination of superstimulation protocols, donor mating management, and embryo collection timing is necessary to elucidate these variations. The effect of recipient health and nutrition on pregnancy maintenance also needs further investigation. Further research in and understanding of reproductive physiology in camelids will provide a better understanding of the production of embryos as well as the interaction of embryos within the uterus prior to collection and following transfer. As further research and clinical data are evaluated, camelid embryo transfer programs will become more efficient and advantageous.

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Nutrition's impact on dairy reproduction
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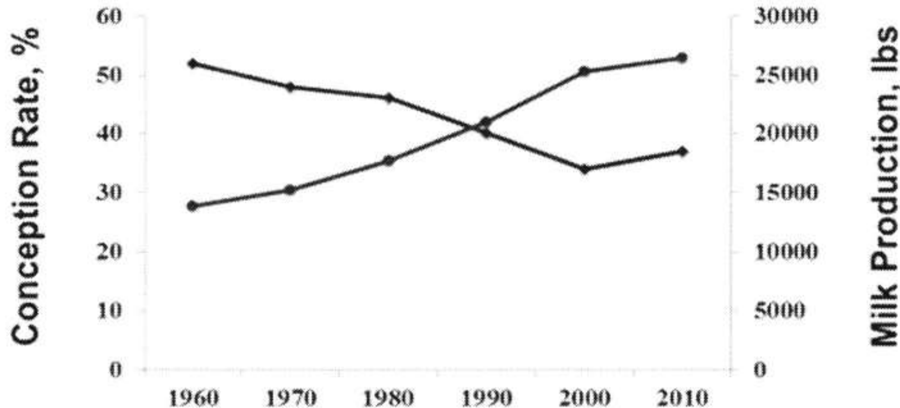


Common nutritional deficiencies and how they affect bovine reproduction

Dry matter intake (DMI) is probably the most essential factor for managing reproduction in postpartum cows.[1] The following reviews several critical components of DMI management that require close attention:

Rumen acidosis

Undoubtedly the most common health problem for most dairy herds, rumen acidosis stems from multiple factors and is characterized by a rumen pH of less than 6.0. The low pH results from excessive lactic and propionic acid produced by the rumen microflora, relative to buffering by bicarbonate-containing saliva produced during cud chewing. The acids damage papillae that line the rumen wall, allowing bacteria to escape into the bloodstream. These bacteria settle in vascular areas where they become trapped. They cause significant pathology, forming abscesses in the liver, lungs, hoof lamina, and subcutaneous tissue.

Due to histamine-induced laminitis and sole abscesses, the affected cows become lame, compounding their health problems. Lame cows often eat poorly, lose body condition, and become more susceptible to other conditions. They may also experience a delayed estrous cycle, which may cause anestrus and delayed conception.

In the event a cow with rumen acidosis exhibits estrus and conceives, the blastocyst is often smaller than normal at nine to 12 days, resulting in early attrition, often referred to as early embryonic death.

Several management tools can help prevent rumen acidosis. These include:

- Ascertaining moisture content of the total ration to prevent sorting by the cows
- Determining particle size of the ration with the Penn State Particle Separator Box
- Providing optimum cow comfort in the heat of summer

For complete information on rumen acidosis prevention, dairy producers should consult with a nutritionist or veterinarian who provides nutritional services.

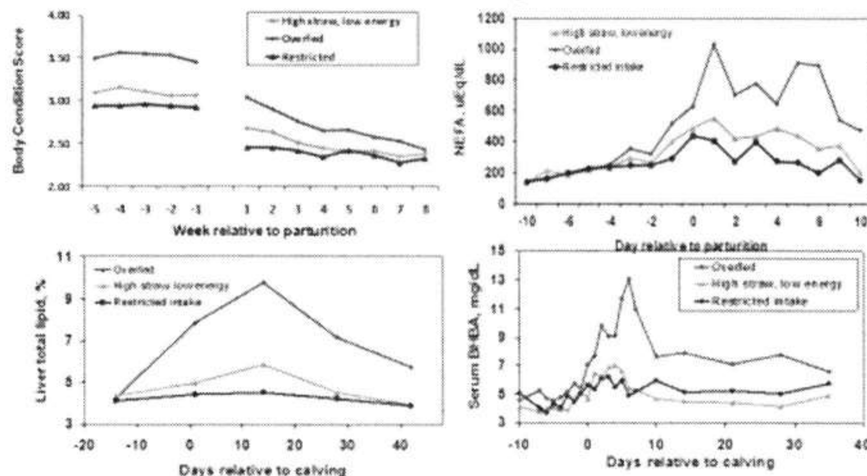
Energy management and/or high body condition score

Excessive condition during the dry period often results in fatty infiltration of the liver and other internal organs, which become surrounded by adipose tissue – even when a cow's body condition score may be considered optimum, at 3.5. Fatty infiltration of the liver, which can also occur rapidly in the last few days prior to calving, may result in development of fewer pre-freshening follicles. Transition dry cows should be supplied a dietary energy maintenance level of 1.3 MegCal/kg,[2] in contrast to a lactating cow receiving 1.65 MegCal/kg. of energy.

A herd with an ineffective transition dry cow program is easy to identify. The cows close to calving often have high body condition scores and are commonly diagnosed with "fat cow syndrome" after calving. Cows that survive being too fat at calving often develop extremely low body condition scores a few weeks later. This results in low milk production, subsequent anestrus, and lower fertility.

Rumen-protected choline and niacin are useful products to add to the transition TMR and the early lactation TMR to aid in metabolizing excess abdominal fat. Positive ramifications of choline supplementation will be discussed further in this paper.

Controlled Energy Diets Prepartum Resulted in Better Metabolic Status Postpartum



University of Illinois at Urbana-Champaign

Janovick and Drackley, 2010

Dietary cation-anion difference (DCAD)

Dietary cation-anion difference in transition dairy cows' diets during the last three weeks before calving is one of the most recently developed tools to help cows calve and lactate successfully. The DCAD formula for determination is ($K^+ + Na^+ - Cl^- + SO_4^-$). This is a method for creating metabolic acidosis in a cow by causing the DCAD to stay in a range between -5 and -15.

Cows normally have a blood pH of 7.4. Excessive anionic chlorides without cations and sulfates drops the blood pH, creating acidosis. The cow's metabolic system responds by pulling calcium from the bones to stabilize blood pH. Increasing the blood calcium level to 8.0-10.0 mg/dl has many benefits. It prevents clinical and subclinical hypocalcemia. If either of these conditions occurs, a cow is susceptible to retained placenta, ketosis, and displaced abomasum. In addition, her immune response is depressed, resulting in increased susceptibility to metritis and mastitis immediately after calving. Calcium is integral to maintaining a strong immune response. It is also essential for muscle function and bone integrity.[3]

A cow near calving requires 0.8% calcium in her diet, when no modifications are made for an anionic diet. Close-up cows (last 21 days prior to calving) should receive 1.0% calcium, provided that an anionic salt is included for a calculated DCAD of -5 to -15. Weekly monitoring of urine pH is essential to

ensure acidosis occurs with a urine pH of 5.6-6.8. Be aware that a pH outside this range may lead to metabolic disorders.

Inadequate calcium in the diet, irrespective of the DCAD, results in reduced immunity and poor muscle tone, especially in the smooth muscles of the abomasum and uterus. Uterine immunity is as essential as smooth muscle tone.

Subclinical low blood calcium of 7.5 or lower causes a reduced defense mechanism in the uterus. This leads to more profound issues with puerperal metritis. Treatment of sub-hypocalcemia may be corrected by intravenous administration of calcium borogluconate or calcium boluses. Acute hypocalcemia is most effectively remedied by intravenous calcium. When subclinical hypocalcemia is diagnosed, an evaluation of the diet is advised to determine changes made in forage calcium values to adjust the DCAD. The ration DCAD should be modified to -5 to -15, using an anionic salt such as BioChlor™, Animate™, or SoyChlor™.

To summarize the overall nutritional strategy for managing hypocalcemia and immune responses: Lower the energy level of the ration by incorporating 3-5 kg of wheat straw/cow/day, chopped at 2.0-3.5 cm and blended with the remaining ingredients. Provide no more than a kilogram of corn and minerals to supply a DCAD of -5 to -15, a vitamin-mineral mix to fulfill the nutritional requirements, 0.1 kg of rumen bypass fat and 14% crude protein for a total mix ration (TMR). No high potassium legume haylage should be fed, unless the DCAD can be maintained within the optimum negative DCAD range. Eight to ten kilograms of corn silage should be included. The TMR should be blended and fed fresh each day to promote full consumption. Large breed dairy cows often consume 13 kg of the transition cow TMR daily. In contrast, cows consume 9-10 kg of feed daily when not on a low energy anionic diet. The major contrast between the transition diet and a lactating diet is reduced energy (close-up cows on TMR consume 1.30 MegCal/kg as compared to lactating cows consuming 1.65 MegCal/kg). This encourages more dry matter consumption by late pregnancy cows.

Phosphorus deficiency and reproduction

Phosphorus (P) is intricately related to many metabolic mechanisms in the cow, such as the workings of the calcium-to-phosphorus ratio.

Historically, soils were low in P, but not so much today. Fertilization practices and advanced crop production methods have eliminated low P levels in forages fed to cows. This issue is referred to as "legacy phosphorus." Many farms have an abundance of soil P. This results in winter run-off into streams and lakes, promoting algae blooms that create oxygen "dead spots" in bodies of water.

Phosphorus is a key element in many enzymatic and endocrine processes including trace mineral absorption and utilization. It plays an integral role with calcium in bone growth and maintenance.

The bovine requirement for dietary P is 0.35-0.40%. Many ruminant diets have a surplus of P. Phosphorus deficiency is rarely diagnosed today. If P deficiency occurs, it manifests as anestrus or at least "silent" estrus. In rare instances, P deficiency plays a role in down cows, appearing as clinical hypocalcemia. A relatively rare condition of acute P deficiency is described as post-parturient hemoglobinuria.

A diagnosis of P deficiency is made with forage and concentrate analyses and a calculation of the cow's dietary intake. Blood tests are mostly ineffectual for diagnosing P deficiency. Correcting the ration formulations generally resolves issues related to low dietary P.

Copper deficiency

Copper deficiency and toxicity present an enigma. The best illustration of this is copper's relationship to molybdenum when fed to sheep. When seven mg/kg of dietary copper are fed to sheep with an inherent level of molybenum under 1.5 mg/kg, toxicity results. This leads to a hemolytic crisis and potential death. In contrast, feeding seven mg/kg of dietary copper to sheep with dietary molybdenum of more than 1.5 mg/kg will cause sheep to manifest copper deficiency signs. While cattle aren't as sensitive to toxicity or deficiency as sheep, a similar paradox exists. Jersey cattle are similar to

sheep in regard to dietary copper and molybdenum. Adult Jerseys should not be fed more than 15 mg/kg of dietary copper unless dietary molybdenum levels are elevated.

Copper status in the bovine affects several enzyme systems. Copper-containing ceruloplasmin is a key enzyme for the synthesis of reticulocytes. Low levels of ceruloplasmin result in anemia. Copper is essential for the enzyme system lysyl oxidase, also known as amine oxidase. Lysyl oxidase is essential for the cartilaginous matrix in which bone osteoblasts lay calcium to provide structural rigidity to bone. A lack of copper causes a loss of integrity in the cartilaginous matrix. This makes young calves susceptible to long bone fractures. This enzymatic system is also necessary for the cartilaginous matrix in the hoof. Deficiency causes heel cracks to develop, resulting in lameness.

Lysyl oxidase plays a key role in maintaining myocardial fibrils. Deficiency during cardiac muscle fiber maintenance results in fibrotic tissue replacing myofibrils, causing what appear to be heart attacks. This malady is often referred to as "falling disease," because dairymen report perfectly healthy cows falling over dead. Observed more often in lambs than calves, amine oxidase is essential for the formation of elastin in the arterial intima of the great vessels such as the aorta and pulmonary arteries. These animals develop aortic or pulmonary aneurysms because of defective elastin in the arterial walls and are diagnosed at necropsy with a blown aneurysm.

Copper is essential to the pigment enzyme tyrosinase, which converts tyrosine to melanin in the black hair of cattle. Copper deficiency causes the hair to fade and appear reddish, as though it will soon shed.

Another copper-containing enzyme is cytochrome oxidase, which is essential for myelination of the brain stem and spinal cord. Termed "a-myelination," it is similar to demyelinating diseases, but in this case, affected calves are born of copper-deficient dams. Such calves are unable to nurse and often have a "star-gazing" look. In my experience these calves never learn to nurse, and in the case of beef calves, never manage to find the dam's teat. This is often a herd problem.

Finally, animals affected by copper deficiency often have a reduced defense mechanism. The mechanism at work here has not been fully described, but one biological system involved relates to Cu,Zn superoxide dismutase. Copper-zinc superoxide dismutase is an intracellular system that is essential in reducing free oxygen radicals as they are metabolically produced. Copper-zinc superoxide dismutase reduces free oxidative radicals intracellularly to hydrogen peroxide. Hydrogen peroxide is then further reduced by selenium-dependent glutathione peroxidase to water and oxygen. Selenium will be addressed further later in this paper. Vitamin E also can and does reduce hydrogen peroxide, similar to Cu,Zn superoxide dismutase.

Several interactions with copper can interfere with copper availability. These include iron, sulfates/molybdenum, and zinc. (See Nutritional toxicities and reproduction, Sanders, SFT Annual Conference 2017.)

The copper nutritional requirement depends upon many other interactions, varying with the presence and level of other minerals. As mentioned previously, sulfate and molybdenum nutritional levels greatly impact the requirement for dietary copper. Requirements: sheep, ~7 ppm; Jerseys, 10-15 mg/kg; and other dairy breeds, 15-25 mg/kg, depending on Mo status when above 1.5 mg/kg and sulfates in water when above 350 mg/kg. Treatment of deficiency includes a Multimin™90 injection and correction of dietary levels with supplementation of chelated copper sources or copper sulfate. Copper oxide is not an acceptable source of copper because of its low biological availability.

Manganese deficiency

Manganese (Mn) typically is not included in a differential diagnosis list because Mn deficiency is considered rare. However, glyphosate herbicide has been reported to reduce the uptake of Mn by row crops. In a couple of instances, this author has confirmed Mn deficiency in swine fed GMO corn, although the GMO corn was not validated to be the cause of the Mn deficiency.

Manganese has also been identified as an element that may be incorporated into the superoxide dismutase intracellular system for scavenging.[4] Diagnosis of Mn deficiency, in this author's opinion, is

difficult because confirmed serum deficiency levels of 0.005 mg/kg[4] are at the low end of the spectrum detectable in a laboratory.

Signs of Mn deficiency include silent estrus, reduced conception, abortions, reduced birth weight, skeletal changes in growing calves, weak calves, and cystic ovaries. Calcium, cadmium, cobalt, iron, and phosphorus are antagonistic to Mn. High Mn causes calcium retention in the fetus. Mn deficiency is rarely diagnosed, yet when manganese assays are performed, manganese frequently is in the range considered deficient.[6]

Manganese uptake naturally occurs from the soil, but is reduced by soil liming. Manganese sulfate is 100% available. The requirement in cattle is 40-200 mg/kg. The treatment of Mn deficiency is readily resolved with Multimin™90 injections and increased dietary $MnSO_4$ to 40-200 mg/kg.

Zinc deficiency

Zinc is linked closely with Cu in superoxide dismutase, to capture free oxygen radicals for conversion to hydrogen peroxide. Zinc is also important for hoof and skin integrity and integral to more than 200 proteins and zinc metallothionein enzyme systems.[5] Parakeratosis is common in zinc-deficient calves and causes stiffness and shortening of bones, increased pododermatitis, reduced conception rate, severely impaired spermatozoan maturation, reduced feed intake, and growth rate, apparently related to reduced insulin levels. A genetic defect in Holsteins (A46) causes inability to absorb Zn from the intestine

Diagnosis of deficiency is readily performed through blood serum tests. Levels of 0.20-0.40 mg/kg are deficient with test reports under 0.60mg/kg considered deficient. (Special blood tubes should be used for mineral assays, as the rubber stoppers of conventional tubes contain zinc, skewing the results.)

Excessive zinc reduces calcium metabolism and vice versa. Copper and iron are also antagonistic. In addition, low zinc inhibits vitamin A absorption, low P increases tissue Zn, high Al reduces Zn absorption, and monensin enhances Zn absorption.

Good sources of supplementation include Multimin™90 injectable, Zn oxide, Zn sulfate, and Zn methionine.

Vitamin E/selenium deficiency

Vitamin E, as alpha-Tocopherol, has long been noted to be closely interactive with selenium. While one can replace most of the other in muscle-related activities, this is not totally the case for other metabolic activity. Vitamin E and selenium perform related activities in the muscle action cycle. Selenium is a component of selenium-dependent glutathione peroxidase (GSH-Px), acting as an antioxidant during release of energy for muscle action. It reduces hydrogen peroxide radicals from Cu,Zn superoxide dismutase to water and oxygen and stimulates production of immunoglobulin M (IgM) antibody, producing cells that positively impact the immune system.

Selenium and vitamin E perform related functions in the muscles. One can replace the other, to an extent. Selenium is an essential element in production of an essential amino acid, cysteine. Selenium is mostly deficient in 44 states, but toxicity is possible in some areas in the western U.S. where soils and selenium accumulator plants contain high levels of selenium.

Injectable forms of vitamin E and selenium include Bo-Se™, Mu-Se™, and Multimin™90 (contains Se but no vitamin E). Dietary sources include inorganic sodium selenite-90, sodium selenite-200, selenomethionine, and sodium selenate. The requirement is 4-6 mg of Se per day for an adult dairy cow, 1,000-2,000 IU/day of vitamin E daily, and 4,000-6,000 IU of injectable vitamin E at calving is very useful.

Signs of deficiency include white muscle disease/cardiac failure in calves, abomasal ulcers, elevated SCC, cystic ovaries, reduced fertility, retained placenta, and oxidized flavor to milk.[6]

Selenium deficiency can be diagnosed through several tests including serum selenium (0.80.300 mg/kg, whole blood selenium 0.200-1.200 mg/kg, Se-GSH-Px 19.0-36.0 *u*moles/min. at 37°/mg Hb). A diagnosis of vitamin E deficiency can be confirmed with serum vitamin E. Normal values are 300-1,000 *u*g/dl for adult cows.

Treatment of selenium deficiency in young calves is by injectable Bo-Se; in adults, with Mu-Se; or in the diet, with sodium selenite 90 mg/lb or selenium chelates. Adult dairy cows benefit from injectable vitamin E or 1,000-2,000 IU/day of dietary vitamin E supplementation.

Vitamin A deficiency

Vitamin A is a key element in colostrum, as the newborn has no reserve vitamin A stores at birth. Vitamin A is needed for growth, appetite, prevention of night blindness, follicular/testicular development, cell replication, and maintenance of epithelia integrity and mucous secretions.[7]

Several conditions affect vitamin A availability. Zinc deficiency and high dietary Ca negatively affect vitamin A utilization, as do increased nitrates in drought conditions and high ambient temperatures. Salt and urea reduce stability of vitamin A supplements, and long storage periods adversely affect vitamin A stability and utilization.

B-carotene from green leafy forages such as alfalfa is a precursor of vitamin A. One milligram of *B*-carotene is equivalent to 400 IU of vitamin A. Other sources of vitamin A include vitamin A retinyl palmitate and injectable vitamin A-D.[7]

The daily requirement for vitamin A in dry cows is 60,000-100,000 IU, and for lactating cows, 53,000-173,000 IU. Supplying twice the recommended dietary level can help compensate for interfering factors and negative interactions.

Signs of vitamin A deficiency include abortions, still births, weak calves, retained placenta, reduced fertility in bulls, reduced conception in cows, poor appetite, reduced weight gains, rough hair coat, reduced defense mechanism, increased pneumonia rate, watery eyes, blindness or night blindness, diarrhea, and swollen joints or brisket.

Deficiency can be diagnosed with the incidence of night blindness, decreased appetite, poor growth, dermatosis, and pneumonia. Adult cattle may have anasarca, mastitis, infertility, and night blindness. Analysis of serum vitamin A is useful for a diagnosis, but must be conducted within one hour of a blood draw – which precludes this option for most diagnosticians.

Treatment of vitamin A deficiency is most easily managed with injectable vitamin A-D. Raising dietary vitamin A levels also is advantageous.

Choline

Little historic research supports the importance of choline for cows. However, this is changing. Rumen-protected choline (RPC) is believed to be liver and calcium sparing,[8] playing a role in increasing fat metabolism.

Rumen-protected choline is highly recommended, as choline must be protected from rumen microbes. Cows can be supplemented during the transition and fresh periods with a commercially available feed additive such as ReaShure® from Balchem.

Data suggest that 15 gm/cow/day of RPC will provide a positive 2 kg/day response in milk production with less ketosis.[9]

Heavy conditioned cows have lower milk production and higher incidence of ketosis. Fat cow syndrome is considered a classic symptom of choline deficiency.[10] Energy should be fed at minimum maintenance levels of 1.3 MegCal/kg. During transition time and early lactation, cows should be fed a DCAD diet and RPC at 15 gm/cow/day.

Choline-supplemented cows have higher IgG levels in their colostrum. These cows also have a statistically significant higher level of blood calcium. Heifer calves born from RPC-supplemented cows were 31 lb. heavier at 12 months of age (even though they were smaller at birth ($P < 0.05$).[9]

Methionine

Methionine is known to interact with rumen-protected lysine with an ideal ratio for optimum performance of 3:1, rumen-protected lysine (RPL) to rumen-protected methionine (RPM). Methionine increases lipid content of blastocyst when fed three weeks prepartum to 30 days postpartum.[11]

Methionine is closely related to choline. It has been observed that choline can spare methionine and methionine can spare choline in transition cows. However, feeding methionine without choline will not aid in the prevention of fatty liver degeneration. Two products that provide rumen-protected methionine are Smartamine™ and Megalac plus™.

Signs of methionine deficiency are subtle. They include a lower milk protein test and an increased rate of early embryonic death. Nutrition modeling software is used to diagnose deficiency. A deficiency is corrected by feeding methionine in the transition ration, continued for 60 days after calving.

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Common nutritional toxicities that impact bovine reproduction

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Mycotoxins

These are a group of the most common toxicities affecting bovine reproduction. Caused by molds and fungi, they are found in feedstuffs. They develop in crops in the field and in storage. The most common examples are zeralanone, deoxynivalenol (vomitoxin), and T-2 toxin. Many other types exist, but these three are likely to have the greatest impact on reproduction. Nonetheless, routine diagnostic testing should cover 20 or more different mycotoxins to determine which are at work.

Mycotoxins date back to the Dark Ages, when *Claviceps* grew on cereal grain crops, producing ergot, which afflicted humans with gangrene and convulsions and caused animals to slough their hooves and abort.

Any one of these toxins extracted from a crop and experimentally added to a diet causes less severe symptoms than mycotoxins that grow naturally in feedstuffs. Scientists postulate that more significant symptoms result from synergistic action with other toxic agents in the diet.

Mycotoxins that negatively affect reproduction are most likely to come from ensiled forages, corn, and small grains.

Signs of mycotoxin toxicity include reduced feed consumption, gastroenteritis, diarrhea, lower milk production, reduced reproductive performance, cystic ovaries, abortions, and, in extreme cases, death. A confirmed diagnosis depends upon multiple clinical signs, laboratory tests of multiple feed samplings, and direct microscopic analysis of feedstuffs to identify mold such as *Aspergillus*, *Fusarium*, or *Penicillium*.

Mycotoxins may occur in isolated pockets of a feed storage facility, depending on the nature of the storage system, areas of oxygen exposure, crop quality in the field, processing of the feedstuff, and moisture content. Because of this, sampling errors may lead to a negative laboratory analysis even though toxin is present in unsampled locations of the storage facility.

The ideal management response to a confirmed diagnosis of mycotoxicosis is to remove the toxin-contaminated feed from the diet or to at least dilute the mycotoxin-carrying feedstuff. Toxin binders such as bentonite, Neutrotox™, or Omnigen™ are another solution. Omnigen™ also has positive neutrogenic effects. No antidotes to these toxic agents exist.

Molybdenum

Molybdenum (Mo) is part of an enigma related to copper and cobalt utilization. Molybdenum uptake by forage plants increases in soils with pH over 6.7.[1] High soil pH also decreases the uptake of cobalt (Co) by forage plants. The increased uptake of Mo at higher soil pH combined with available sulfur creates a biologically active complex, thiomolybdate. Thiomolybdate binds copper (Cu) in the gastrointestinal tract, preventing absorption. High soil pH, while causing higher Mo uptake, simultaneously reduces Co uptake. So, a dual deficiency of Cu and Co is common.

This conundrum results often in legume forages with high organic content, high pH soils, and aggressive soil liming.

Bovines require small amounts of Mo[2-4] as an essential component of xanthine oxidase, aldehyde oxidase, and sulphite oxidase,[3] which are involved in intracellular ATP utilization.

The pre-eminent indication of Mo toxicity is a secondary Cu deficiency with a primary Co deficiency. Molybdenum levels above 1.5 mg/kg, with increased incidence of ketosis, is considered the threshold for the interaction with Cu and Co.

Treatment options include injectable Multimin™. Neutralize the effects of Mo toxicity by raising dietary copper levels with a chelated copper source or copper sulfate. The level should be adjusted from a range of 6:1 to 8:1 Cu:Mo ratio, plus 10 mg/kg base supplementation. Use B_{12} injections after calving for cows exhibiting ketosis caused by cobalt/vitamin B_{12} deficiency.

High blood urea nitrogen (BUN)

Dairy cows, because of high milk production, have a higher protein requirement than beef cows or dairy heifers. By having a rumen, cows utilize rumen degradable protein (RDP) and rumen undegradable protein (RUP) for maintenance, growth, and milk production. A 60:40 RDP-to-RUP ratio is recommended. Water is the first limiting nutrient of ultimate importance, just ahead of the energy requirement. Energy is necessary for the rumen microflora to break down RDP into ammonia, then re-assimilate it into usable amino acids for absorption in the abomasum and the small intestine.[5]

Diets with excessive RDP and/or inadequate energy reduce the rumen microflora's ability to efficiently re-assimilate ammonia into amino acids. This causes ammonia to escape through the rumen wall into the portal blood stream. The liver conjugates ammonia to plasma urea nitrogen (PUN), which causes higher blood PUN. When ammonia concentration exceeds the liver's capacity, excess ammonia can also build up in tissues including the uterus and ovaries.

Plasma urea nitrogen directly correlates to milk urea nitrogen (MUN). High PUN also increases urea and ammonia in follicular fluid. This causes lower embryo cellular cleavage rates. Milk urea nitrogen analysis is a very inexpensive test readily available through Dairy Herd Improvement (DHI) record systems and can also be sampled from bulk milk. Generally, MUN levels should be below 14 mg/dl. Levels of >16.0 is a red flag that PUN is negatively affecting reproductive performance.

One elevated herd bulk milk tank or DHI herd analysis, however, should rarely be cause for action. The diagnostician should check monthly for a trend in PUN.

Cows on high protein diets (23%) or exclusively legume forage diets have higher urea nitrogen in uterine fluid than cows on 12% crude protein (CP) rations. Uterine pH normally increases from 6.8 to 7.1 on day seven of the estrous cycle (luteal phase), but pH does not increase when cows have excessive RDP.[6] Pregnancy rates are lower in recipient heifers after transfer of embryos recovered from high PUN cows vs. moderate PUN cows (11% vs. 35%). Urea acts on oocyte maturation but does not impede the developing embryo.[7] Pregnancy rates are not affected by high PUN when good quality embryos from normal PUN dams are transferred to recipients with high PUN.[8]

A diagnosis of the effect of PUN on pregnancy rates must be based on PUN or MUN analysis and a ration analysis. The ration analysis should include CP and predictions of RDP and RUP, as contrasted to dietary energy. A nutritional modeling program is helpful in ration evaluation.

Treatment and prevention of a negative reproductive impact caused by dietary protein requires the ration to be reformulated to lower CP and RDP and correlate them to energy.

Subclinical nitrate toxicity

Nitrates are noted for causing several rare, severe health conditions in ruminants and, at high levels, even death. The rare but acute form of nitrate toxicity is most common from consuming nitrate fertilizers, grazing heavily fertilized fields, or drinking runoff from heavily fertilized fields. Plants such as *Brassica,* cereal grains, or sweet clover may also contain high levels of nitrates. The purpose of this discussion is to describe *subclinical* cases of nitrate toxicity rather than clinical life-threatening cases. In acute cases, symptoms include abdominal pain, weakness, drooling, blue discoloration of the mouth, mouth breathing, collapse, coma, and death,[9] though not with lower levels of dietary nitrates.

Cows fed high nitrate corn silage (0.78% NO_3) as half their total digestible nutrient do not demonstrate clinical signs of nitrate toxicity. They also exhibited no noticeable effect on vitamin A levels, milk production, or reproduction.[10]

Nonetheless, low level nitrate toxicity may occur in certain instances. Pubertal heifers fed daily diets containing 0, 440, and 660 mg/kg of body weight, starting three estrous cycles prior to breeding, were impacted at the highest dietary level.[11] Conception rate was lower in heifers fed 660 mg of dietary nitrates. One abortion occurred in heifers fed the lowest level of nitrates (which was not significant). Two abortions and two deaths occurred in heifers on the highest dietary level of nitrates. There were no differences in estrous cycles, length of gestation, birth weight, or performance of the calves. Vitamin A and carotene levels were unaffected. Milk production postpartum was the same for all groups.

Gossypol toxicity

Gossypol is a naturally occurring phenolic compound produced by pigment glands in cotton stems, leaves, seeds, flower buds and also contained in cottonseed meal, hulls, and cottonseed cake. Gossypol is toxic to most monogastric animals, but somewhat less so to ruminants because of their ability to detoxify it in the rumen. In the bovine, gossypol-containing cottonseed and other cottonseed products cause a male fertility issue at dietary gossypol levels as low as eight grams per day. It appears dairy cows are not affected until approaching 20 gm/day of gossypol consumed orally.

Gossypol levels vary depending upon the extracted product produced. For instance, cottonseed hulls have much safer and lower levels than cottonseed cake.

Signs of toxicity first appear in bulls on a whole cottonseed diet. A bull can be rendered infertile within a couple of months of consuming no more than 2.5 kg of cottonseed containing as little as one percent gossypol.

Quantity purchases of whole cottonseed for dairy cattle should be made contingent on analysis of the gossypol content. If not being provided to bulls, whole cottonseed fed to cows is acceptable with a level of two percent gossypol. The diet for the cows should not exceed 2.5 kg/hd/day. If the whole cottonseed exceeds 12 gm/cow/day, vigilance is needed. It has been confirmed that 20 gm/cow/day of gossypol may be fatal to dairy cows.

Natural plant toxicities

A number of natural plant toxins may also impact the bovine species. Many bovines, however, will not touch these plants until pastures are short and they are searching for something to eat. The following tables categorize these natural plant toxins.

Table 1. Natural toxins affecting embryonic and fetal development

Toxic Plant	Toxin	Effect	Reference
Veratrum californicum (False hellebore)	Jervine, cyclopamine	Facial & skeletal defects Tracheal stenosis	James et.al., 1992
Lupinus spp. Lupine	Anagyrine quinolizidine alkaloid	Cleft palate	James et. al. 1992
Poison hemlock	Piperidine alkaloids	Skeletal defects	James et.al. 1992
Locoweed	Indolizidine alkaloid	Fetal edema, enlarged rt. heart ventricle	
Tree tobacco	Piperidine alkaloid	Skeletal defects, cleft palate	James et.al. 1992
Selenium-accumulators	Selenium	Deformed hooves	James et.al. 1992
Ponderosa pine	unknown	Light birth weight	James et.al. 1992

Table 2. Plants causing abortion or embryonic deaths in livestock

Plant	Toxin	Effect	Reference
Locoweed	Indolizidine alkaloid	Abortion, embryonic death Delayed placentation	James et. al. 1992
Broom snakeweed	unknown	Abortion, premature birth	Kingsbury, 1984
Ponderosa pine	unknown	Abortion, premature birth	James et.al. 1989
Little leaf horsebrush	unknown	Abortion	Johnson, 1974
Veratrum californicum	Jervine	Embryonic & fetal death	Binns et.al. 1963

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Effects of age and metabolic disease on the equine oocyte and early embryo

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Abstract

Factors that alter oocyte and embryo viability impact the potential for offspring production. One of the most consistent aspects affecting fertility across species is maternal aging. With advanced age, mares have reduced pregnancy rates, increased pregnancy losses, and lower foaling rates. Problems associated with the uterus and posterior reproductive tract are more prevalent in older mares; however, embryo developmental potential is also impacted prior to the oviductal embryo's entry into the uterus. Ultimately, maternal aging has a deleterious impact on oocyte quality and the ability of older mares to produce offspring. While the overweight or obese mare has historically not been considered a fertility problem, more recent studies have demonstrated weight-associated alterations in cycle characteristics and ovulation. In addition, excess weight in the mare is associated with alterations in composition of the oocyte and developing embryo, suggesting that the body condition of the mare can have long-term implications on the offspring. We will review previous research and highlight new findings regarding alterations in the oocyte and embryo that are associated with maternal aging and weight.

Keywords: Mare, equine, oocyte, embryo, age, metabolism, weight

Introduction

In addition to genetics, the oocyte provides components critical for the successful completion of fertilization and early embryo development. The maternal-embryonic transition, with activation of the embryonic genome and associated transcription, has been determined to occur by the third cell cycle of the equine embryo.[1] Until that time, the early conceptus relies on the oocyte for proteins and mRNAs produced during the oocyte growth phase.[2] Mitochondria are also maternally inherited, and the developing embryo initially relies on mitochondria that originated in the oocyte.[3] Therefore, many of the essential functions of the early embryo are dependent on the oocyte.

Fertilization and the first stages of embryo development occur in the oviduct. The equine embryo remains within the oviduct a relatively long interval, reaching the uterus approximately 5.5 to 6 days after fertilization and at the morula or early blastocyst stage of development.[4] The uterotubal junction in the mare provides a barrier between the uterus and oviduct, potentially limiting the effect of uterine pathology on the oviductal milieu and developing embryo. After passage through the oviduct, the embryo is exposed to the uterine environment. Embryos for transfer are typically collected by uterine lavage at 7 or 8 days after ovulation. By this time, multiple factors can affect the embryo's future developmental potential, including handling, uterine environment, and timing of fertilization. However, less obvious factors which impact oocyte viability can also have a profound influence on the potential for a given oocyte or embryo to develop into a healthy offspring. Two such potential factors are mare age and excess weight. The impact of metabolic status and maternal aging will be reviewed as it relates to the equine oocyte and early embryo development.

Metabolic status

Historically, relatively minimal research has been associated with mare weight and the implications on maternal fertility and offspring health, although increasing attention is being given to this area in human medicine. In recent years, some research has been conducted in horses to try and determine if and how obesity affects reproduction. Increased adiposity in mares is associated with alterations in the estrous cycle and intrafollicular environment. Obesity in mares is correlated with decreased insulin sensitivity, elevated systemic concentrations of insulin, and increased tumor necrosis factor-α (TNFα), and these changes are exacerbated by mare age.[5] Obese mares tend to have extended interovulatory intervals with lengthy periods of elevated circulating concentrations of progesterone

caused by the formation of persistent anovulatory follicles.[6] Induction of transient insulin resistance in mares of normal body weight also results in lengthened interovulatory intervals; however, circulating concentrations of luteinizing hormone (LH) and follicle stimulating hormone (FSH) are not affected. This suggests that metabolic changes, including increased insulin, directly affect the ovary rather than the hypothalamo-pituitary axis.[7] Increased insulin can also disrupt granulosa cell gene expression of the collagenase system both in vitro[8] and in vivo.[9] The collagenase system is an essential part of ovulation,[10] and an inability of collagenase to function normally could contribute to formation of persistent anovulatory follicles or disrupt the timing between meiosis resumption of the oocyte and ovulation. Preantral follicles when cultured in supraphysiological concentrations of insulin have increased production of reactive oxygen species and lower survivability than follicles cultured in lower physiological concentrations.[11] The extent that factors other than insulin affect ovarian function is still being investigated.

Obesity in mares is associated with disruptions in the follicular milieu. Follicular fluid from obese mares has elevated insulin and leptin and tends to have higher concentrations of triglycerides and cholesterol.[12] Granulosa and cumulus cells from these follicles have altered expression of genes involved in lipid homeostasis and endoplasmic reticulum, mitochondrial and oxidative stress.[12] Similar to metabolic syndrome in humans, equine metabolic syndrome (EMS) is a clinical condition associated with obesity or regional adiposity, insulin resistance, and a predisposition for laminitis.[13] Mares with EMS, even in the absence of an obese body condition, have disrupted follicular environments when compared to controls of similar ages and types. Mares with EMS have increased concentrations of insulin and leptin and decreased concentrations of adiponectin in their follicular fluid.[14] Mares with EMS also have increased inflammatory cytokines, TNFα and IL-1β, in follicular fluid.[14] The studies confirm that obesity in mares impacts the follicular milieu in which the oocyte is housed.

The impact of maternal weight on the equine oocyte and embryo has just recently been investigated. Oocytes were collected from mares classified as normal weight or obese, based on body condition scores (BCS) and body fat (normal, BCS 5.1 and body fat of 10.4% and obese, BCS of 7.9 and body fat of 16.2%), and analyzed. Oocytes from obese mares contained more lipids associated with triglycerides when compared to the oocytes from normal-weight mares which contained more lipids associated with cell membranes (phosphatidylcholines, phosphatidylethanolamines and sphingomyelins).[12] The findings suggest that maternal weight is impacting oocyte composition. In a follow-up study, differences in gene expression were noted between embryos from obese when compared to normal-weight mares. In addition, the endometrium from the obese mares had increased expression of genes associated with inflammation (Bresnahan and Carnevale, unpublished data). Weight-associated alterations in the uterine environment and embryos could influence prenatal programming and result in long-term effects in offspring. This has not been studied in horses, but it has been demonstrated in sheep. Obesity in ewes results in maternal and fetal hyperglycemia and hyperinsulinemia,[15] and lambs born to overfed dams have decreased insulin sensitivity.[16] Children exposed to maternal obesity are also more likely to develop obesity and glucose intolerance.[17] There have been limited studies into the impacts of maternal obesity on foals; however, evaluated high starch diets resulted in trends for both decreased IgG concentrations in colostrum[18] and decreased insulin sensitivity in foals at 160 days of age.[19] Although substantial work is needed in this area, it is feasible that weight management of the broodmare is important for optimal fertility and offspring health. These studies also suggest that nutrition and body condition of the mare is important prior to, as well as after, conception.

Age

Although the inciting causes are different, old mares and metabolic/obese mares share some similarities. Old horses have increased inflammatory cytokine production when compared to young horses, and old horses have a greater increase in cytokine production when they are obese than thin.[20] Weight gain in older horses is positively correlated to increased inflammatory cytokine production.[20] As observed in obesity, granulosa cells from old mares have disruptions in transcription of several genes. Old mares have an increase in gene expression of receptors for insulin and adiponectin and peroxisome

proliferator-activated receptor gamma which could result in altered glucose or fatty acid metabolism within follicles.[21] Old mares also have increased expression of the interleukin-6 system and genes associated with endoplasmic reticulum and mitochondrial stress, which is similar to obese mares.[21]

Aging affects various aspects of reproduction, including ovarian function. As some mares reach their teens or early twenties, the ovaries can become large and very active in appearance; potentially, this is caused by a delay of atresia, growth of more follicles to medium sizes, and smaller preovulatory follicles.[22] As mares get closer to the cessation of reproduction, ovarian follicular activity is reduced, and the number of antral follicles imaged using ultrasound can be minimal.[24] Eventually, ovulation will occur at extended intervals, prior to the total cessation of follicle growth.[23-25] The timing of reproductive senescence is mare dependent, although genetics are likely a mitigating factor.

The impact of mare age on fertility has been well documented. In general, fertility begins to decline as mares reach their teen years, with a progressive decrease as mares enter into their twenties. Embryo transfer has been widely used for older, subfertile mares. In general, embryos collected from the uteri of older mares are more likely to be delayed in development, poorer quality, and less likely to result in pregnancies. In addition, embryo collection rates are lower for older mares. Researchers in the laboratory of Dr. G.L. Woods identified a high embryo loss rate between 2 and 14 days of gestation in older, subfertile mares (mean age of 17 yr) when compared to young, fertile mares (mean age of 5 yr).[26] The research group subsequently collected Day-4 oviductal embryos (Day 0 = ovulation) from similar donor groups (mean ages of 6 and 19 yr) and transferred them into the uteri of normal recipient mares, and the embryo loss rate was again significantly higher for embryos from older, subfertile versus young, fertile mares.[27] The general conclusion was that there were intrinsic defects in the embryos from older, subfertile mares or a deleterious effect of the oviductal environment.

In subsequent research in the laboratory of Dr. O.J. Ginther, oviductal embryos were collected at 1.5 or 3 days from young (2-10 yr) and old (>20 yr) pony mares. When compared to embryos from young mares, morphology scores were poorer for embryos from old mares, and developmental progression was delayed.[28] These results again suggested that fertility was impacted by mare age prior to embryo entry into the uterus. Ultimately, oocyte transfer was used to assess the impact of donor age on oocyte developmental quality. Using this methodology, embryos developing from the oocytes of old mares would have no exposure to the tubular genitalia (uterus or oviduct) that could impact fertilization and embryo development. For the study, oocytes were collected from the maturing follicles of young or old donors and transferred into the oviducts of young, inseminated recipient mares. Fertilization and embryo development occurred in the young mares' reproductive tracts. Regardless, embryo development rates were significantly lower after transfer of oocytes from old versus young donors (31% and 92%, respectively).[29] Results of this study suggested that the oocyte was impacted by maternal aging and that an age-associated loss of oocyte developmental potential was the ultimate cause of fertility failure.

The extent that age-associated changes in oocyte quality are inherent to the gamete versus a result of the follicular environment or hormonal stimulation has been an ongoing question in the mare, as in the woman.[30] Cyclic changes, associated with ovarian follicular activity and systemic concentrations of gonadotropins, occur as mares enter their teen years and are altered by increasing age.[22,23] Reproductive senescence can occur at variable ages in mares; but by the mid-twenties, a subset of mares will not have any ovarian activity;[23,24] this is likely associated with the depletion of oocytes, as documented in women (reviewed in Carnevale 2017[30]) as gonadotropin levels remain sufficient to induce follicle growth.[23] Equine aging also affects temporal and quantitative differences in gene expression for key signaling proteins in the dominant, follicular-phase follicle, suggesting that the oocyte-follicle interaction is impacted with maternal age and that synchronization of oocyte and follicle maturation could be disrupted.[31] Age-associated differences can be observed in the gene expression of follicle cells[21,32,33] and in follicular fluid exosomal microRNAs.[33] In total, these studies demonstrate that age-associated ovarian changes impact follicular dynamics and function. The extent that these changes ultimately impact oocyte/embryo quality and fertility are not known.

Regardless of the inciting cause, mare age affects the developmental potential of oocytes and embryos. Oocytes from old or young mares are capable of fertilization in vivo or in vitro. When

oviductal oocytes/embryos were collected at variable days after insemination and ovulation, fertilization rates did not appear to be different between young and old mares.[26-28] After intracytoplasmic sperm injection, the sperm-injected oocytes of young and old mares had similar cleavage rates; however, pregnancy losses increased in an approximately linear fashion from the youngest to oldest age groups of mares, resulting in an approximately 60% increase in pregnancy losses for mares aged >23 yr versus fertile mares aged 3-13 yr.[34,35] Similarly high embryo loss rates have been observed in vivo for old mares (≥ 15 yr) when compared to young mares (5-7 yr) even after insemination with a fertile stallion and in a controlled research environment.[36] The findings again suggest that oocytes from old mares are capable of fertilization and early cleavage; however, maternal aging is associated with a decline in oocyte developmental capacity and high embryo loss rates, with many of the losses occurring early in gestation.

The determination of what aspects of the equine oocyte are affected by maternal age is ongoing. Morphologic differences have been observed using light microscopy between cumulus-denuded oocytes from young and old mares. The most notable age differences were an increase in the volume within the inner zona pellucida and of the perivitelline space for oocytes from old mares; both of which were associated with lower developmental rates.[37] As ooplasm volume was not different between young and old mare oocytes, the larger, thinner zona pellucidae observed for old mares suggest the oocytes grew to a larger size during follicular development, potentially as a result of alterations in oocyte signaling factors.[31] The most notable difference after imaging of oocytes from young (3-10 yr) and old (>19 yr) mares using transmission electron microscopy was the presence of large vesicles within the ooplasm of old mares' oocytes or other anomalies, such as irregular shape, areas of ooplasm devoid of organelles, and sparse microvilli over portions of the ooplasm.[38] Using confocal microscopy to image oocytes from young mares (4-11 yr) and old mares (≥ 20 yr), oocytes at metaphase II from the older group had a significantly higher incidence of chromosome misalignment at the meiotic spindle.[39] Chromosomal misalignment predisposes oocytes to aneuploidy, which could be a cause of embryo loss. Cytoskeletal alterations have also been observed in the oocytes from old mares,[40] and there is evidence of loss of mitochondria numbers and function as the oocytes from old mares mature.[1,41] While one inciting cause cannot be identified, the oocytes from old mares appear to have morphological and functional changes which can impact their capacity to develop into viable embryos and, ultimately, offspring.

Conclusions

Although a variety of reproductive problems are observed for older mares, aging is also associated with reduced developmental potential of the oocyte and embryo. Causes for this loss of oocyte viability are probably complex and multifactorial. However, combining observations associated with maternal aging in mares and women, it is likely that the oocytes of older mares can be predisposed to aneuploidy, potentially associated with loss of structural integrity and failure of energy production. Ultimately, while most oocytes are capable of undergoing fertilization and early cleavage division, high rates of embryo death occur for the old mare, beginning soon after fertilization and continuing until fetal formation. Maternal weight has been shown to impact cycle characteristics and ovulation. The impact of weight on the developing conceptus and offspring have been studied to a lesser extent. However, we now know that the lipid composition of the oocyte changes with obesity in mares, as well as aspects of embryo development and uterine inflammation. In other species, attention has been focused on the impact of maternal weight and nutrition, not only on fertility and pregnancy, but on the future health of the offspring. This remains to be investigated in the horse for both maternal weight and aging.

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The problem mare: clinical perspectives

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Abstract

A problem mare may be defined as a mare that is not pregnant after being bred to a fertile stallion over three estrous cycles, a mare that cannot successfully carry a foal to term, a mare with known reproductive pathology, or a mare with behavioral issues related to reproduction. Obtaining a complete reproductive history and performing a thorough reproductive evaluation are important in detection of the cause(s) of subfertility or infertility and determination of an optimal therapeutic or management plan.

Keywords: Mare, problem, breeding soundness evaluation

Introduction

Reproductive problems are encountered frequently in broodmare practice. A problem mare may be defined as 1) a mare that is not pregnant after being bred to a fertile stallion over three estrous cycles, 2) a mare that cannot successfully carry a foal to term, 3) a mare with known reproductive pathology, or 4) a mare with behavioral issues related to reproduction. The goal of this review is to provide a clinical perspective to the evaluation and reproductive management of 'problem mares', with a focus on common or challenging ovarian, cervical and uterine abnormalities.

Reproductive problems

A list of the common and less common reproductive problems in broodmares is presented in Table 1.

Reproductive evaluation of problem mares

Identification

All mares should be properly identified, and the registration name, registration number, breed and birth date recorded. Photographs should be taken or accurate drawings of markings and tattoos recorded.

Reproductive history

The reproductive history is one of the most important aspects of understanding a problem mare. A complete breeding history should be obtained, including current reproductive status (maiden, barren, open, pregnant or foaling), number of cycles bred during the last season, date of last breeding, breeding technique used (artificial insemination, natural cover or pasture breeding), semen type (fresh, cooled or frozen), number of stallions used, date of last foal, number of previous foals and any previous history of abnormal estrous cycles, uterine infections, embryonic loss or abortion.

Physical examination

A general physical examination should be performed to assess whether the mare has the capacity to carry a foal to term. The evaluation should include, but is not limited to, examination of the oral cavity, eyes, and respiratory, cardiac and musculoskeletal systems. In addition, diet and body condition should be evaluated. In some cases, mares should be screened for pituitary pars intermedia dysfunction (PPID).

Perineal conformation

The external genitalia (vulva) should be evaluated for conformation and muscular tone. The vulva is the first physical barrier for prevention of contamination of the reproductive tract by pathogenic organisms. The optimal perineal conformation consists of a vulva in a nearly vertical position with at least 70% of the vulva ventral to the brim of the pelvis. The muscular tone of the vulva should be

sufficient to prevent aspiration of air into the vestibule or vagina. Horizontal sloping of the vulva secondary to recession of the anus and/or poor muscular tone to the labia of the vulva may predispose the mare to an ascending infection of the uterus.

Estrus detection

The mare should be teased with a stallion that exhibits good libido in order to successfully evaluate estrous cycle stage. Adequate time should be taken to allow shy or nervous mares to express behavioral estrus. Maiden mares may not show heat well and foaling mares may not show heat unless the foal is restrained and safely away from the stallion. It is not very efficient to tease mares as a group, since often the only mares that may come to the fence or tease rail may be assertive mares in heat or mares that want to attack the stallion. One may not be able to determine the heat status of mares that remain a distance from the stallion. It is generally more effective, but certainly more time consuming to tease mares individually. There are many systems used for teasing mares, including chutes, rails, fences, pens and paddocks. Keys to successful teasing are patience, persistence and knowing the behavioral characteristics of each mare.

Transrectal palpation

The goal of palpation is to identify significant features of the reproductive tract, determine stage of the estrous cycle and identify potential problems. Palpation is often performed in conjunction with transrectal ultrasonography. Manual palpation can identify some features of the tract that cannot be detected by ultrasonography. These include tone in the uterus and cervix, softness of an ovarian follicle, sensitivity of the ovary to touch and pressure, etc. Manual palpation is also valuable in confirming the presence of parovarian cysts.

Transrectal ultrasonography

Ultrasound is used in broodmares to visualize structures in the reproductive tract that cannot be discerned on palpation *per rectum*, in the early diagnosis of pregnancy, diagnosis of twins and evaluation of potential ovarian or uterine pathology. Ultrasound is helpful in differentiation of pathologic conditions of the ovary, such as persistent anovulatory follicles and ovarian tumors, as well as uterine issues such as the presence of free fluid within the uterine lumen and lymphatic cysts.

Vaginal speculum examination

A vaginal speculum examination is performed to evaluate the vaginal vault and the external os of the cervix, determine the stage of the estrous cycle, and to detect pathologic conditions such as urine pooling, cervical discharge and trauma to the cervix and/or vagina. Urine pooling is recognized by an accumulation of cloudy yellow fluid in the anterior vagina. Pooling of urine is most common in older mares with poor perineal conformation and mares in poor body condition. Multiple examinations throughout the estrous cycle may be necessary to detect urine pooling, as some mares only accumulate urine in the vagina during estrus. Relaxation of the cervix during estrus may allow for a direct flow of urine into the uterine lumen, resulting in significant inflammation and a reduction in fertility. Vaginal varicose veins may be very small or up to 5 mm or larger in diameter and are located in the region of the vestibular-vaginal junction. Spontaneous or traumatic rupture of vaginal varicose veins is a source of intermittent bleeding from the vulva.

Digital examination of the cervix

After the speculum examination is completed, the cervix may be examined manually for patency and the presence of abnormalities, such as cervical lacerations or adhesions. In addition, a speculum examination and digital examination can be used to identify older maiden mares that have an abnormally tight cervix when in estrus, which will predispose the mare to persistent endometritis after mating.

Endometrial culture

Culture of the uterine lumen is usually done in conjunction with cytology for the diagnosis of uterine infection. Uterine infection can be suspected in a mare that exhibits an abnormally short estrous cycle, has a vaginal or cervical discharge, an inflamed cervix on speculum examination, fluid in the uterus detected on ultrasound, and possibly failure to become pregnant when bred to a fertile stallion. Infectious endometritis is a significant cause of reproductive inefficiency in broodmares. Options for collection of a sample for culture include a guarded uterine swab and a low volume uterine lavage. The uterine sample can be applied to microbial agar plates (i.e. a Quad Plate containing blood agar, MacConkey agar, plus Gram-positive and Gram-negative chromogenic agars) and cultured in a basic incubator. Bacterial growth is usually evident within 24 to 48 hours. Antibiotic susceptibility tests should be performed for any bacteria cultured, but especially for gram-negative organisms such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*.

Endometrial cytology

Cytology is used in conjunction with culture and biopsy in the diagnosis of endometritis. Samples may be collected using a guarded uterine swab or brush or via a low volume uterine lavage. Presence of more than one to 2 neutrophils per high power field is an indication that significant inflammation is present.

Endometrial biopsy

A uterine biopsy is primarily used as an aid in the diagnosis of uterine pathology and as a prognostic indicator of the ability of a mare to carry a foal to term. It is recommended that biopsy samples be initially placed in Bouin's solution for 24 hours and then transferred into alcohol prior to submission to a diagnostic laboratory. Studies have shown that Bouin's solution preserves bacterial biofilm better than formalin solution.

Hysteroscopy

Evaluation of the uterine lumen using a videoendoscope may be used to detect intrauterine adhesions, cysts, and foreign bodies, as well as inflammation, fibrosis and other abnormalities.

Endocrinology

Analysis of reproductive hormones in the non-pregnant mare is most commonly performed to evaluate corpus luteum function and to diagnose ovarian abnormalities. A progesterone concentration of <1 ng/ml indicates an absence of luteal tissue, while concentrations >1 ng/ml indicate the presence of an active corpus luteum. Testosterone, inhibin and anti-Müllerian hormone are utilized as endocrine markers of the presence of an ovarian granulosa cell tumor (GCT).

Karyotype

Analysis of the chromosome number and structure may be an important diagnostic test in the evaluation of primary infertility or repeated pregnancy loss in mares.

Other tests

In some cases, the examination procedures noted above may not identify the cause of subfertility. Consequently, other diagnostic tests may be indicated (Table 2).

Common or challenging reproductive abnormalities in mares

Failure of follicular development

It is not unusual for mares in late winter or early spring to have minimal follicular development even if they have been under lights for 60 days or more. One option for stimulation of follicular development is twice daily administration of a low dose (i.e. 10 to 125 μg) of a gonadotropin releasing

hormone (GnRH) agonist such as deslorelin, buserelin or histrelin. Mares in spring transition (i.e. with follicles ≥25 mm in diameter) are more likely to respond than mares in deep winter anestrus (i.e. follicles <20 mm in diameter). Mares in deep winter anestrus that do respond to low-dose GnRH agonist therapy are more likely to revert back to anestrus after treatment is discontinued than mares initially treated during spring transition. In general, follicular development is evident within three to five days after the onset of therapy. It is recommended that human chorionic gonadotropin (hCG) be administered to induce ovulation of a follicle stimulated by low-dose GnRH therapy.

Ovulation failure after hCG/deslorelin administration

Induction of ovulation is advantageous if a mare is in a timed breeding, shipped semen, frozen semen or embryo transfer program. Unfortunately, not all mares that are administered hCG or a GnRH agonist (deslorelin, histrelin, etc.) ovulate as predicted. In our clinical experience, approximately 50% of mares that fail to ovulate on schedule after administration one ovulation induction agent will ovulate after administration of the alternative agent during the same estrous period (i.e. administration of hCG if mares fail to ovulate after deslorelin treatment). In contrast, it is rare that a second dosage of the same induction agent will result in ovulation.

Anovulatory follicles

There are two subtypes of anovulatory follicles. Approximately 85% of anovulatory follicles are associated with hemorrhage and eventual luteinization. Hemorrhagic anovulatory follicles (HAF) form when significant bleeding occurs into the lumen of the dominant follicle during estrus. Initially the blood does not clot due to anti-coagulant factors present in equine follicular fluid. Eventually the blood clots and the fibrin scaffolding within the blood clot allows granulosa and theca cells to invade and luteinize. Luteinized anovulatory follicles produce a large amount of progesterone and are eventually responsive to prostaglandin administration.

In contrast, approximately 15 % of anovulatory follicles remain as non-viable persistent anovulatory follicle (PAF). As the follicle becomes non-viable, estrogen levels decrease, uterine edema goes away and the mare goes out of heat. A PAF does not luteinize, does not produce progesterone and will not respond to prostaglandin therapy.

In addition to the two primary subtypes of anovulatory follicles, a low percentage of mares may exhibit partial collapse of a follicle. An initial presumption may be that the mare was examined while in the process of ovulating, but in reality the partially follicle will remain in this state for many days. Pregnancy rates associated with a partially collapsed follicle are low, possibly due to failure of the oocyte to be discharged from the follicle or possibly a deficiency in follicular or oocyte quality.

Hemorrhagic anovulatory follicles contain blood which can be detected ultrasonically as scattered free-floating echogenic spots within the follicular fluid. Ballottement of the follicle will cause the echogenic particles to swirl within the non-clotted hemorrhagic follicular fluid. The follicular fluid will eventually form a gelatinous, hemorrhagic mass within the follicular lumen which may be viewed as echogenic fibrous strands traversing the follicular lumen. A thickening of the follicular wall may be observed in association with luteinization of the follicular wall. Plasma progesterone concentrations are often markedly elevated. In contrast, a PAF will retain a similar ultrasound appearance as a large viable pre-ovulatory follicle. Occasionally a small amount of echogenic particles may be visible in the follicular lumen. A PAF may remain present for several weeks and eventually be replaced by a new dominant follicle.

Luteinized HAF and PAF will both eventually regress spontaneously. A luteinized HAF can be eliminated with a single dose of prostaglandin (i.e. cloprostenol; 250 μg, im) administered nine to ten days after the initial recognition of echogenic particles within the follicular lumen. A PAF will not respond to prostaglandin administration as there is no luteinized tissue present.

Ovarian tumors

The most common ovarian tumor in the mare is the granulosa cell tumor (GCT). Granulosa cell tumors are almost always unilateral, slow growing, and benign. Examination of the affected ovary using transrectal ultrasonography often reveals a multicystic or honeycombed structure, but the tumor may also present as a solid mass or as a single large cyst. The contralateral ovary is usually small and inactive, although mares with a GCT on one ovary and a functional contralateral ovary have been reported. Behavioral abnormalities such as prolonged anestrus, aggressive or stallion-like behavior, and persistent estrus may be expressed in affected mares.

Granulosa cell tumors are hormonally active, and clinical diagnostic assays for the detection of a GCT include the measurement of anti-Müllerian hormone (AMH), inhibin, testosterone, and progesterone (Table 3). Anti-Müllerian hormone has recently been described as the most sensitive indicator of the presence of a granulosa cell tumor, and is elevated in approximately 95 % of mares with histologically confirmed GCT. Inhibin is elevated in approximately 90 % of mares with a GCT. It has been hypothesized that inhibin produced by the GCT is responsible for the inactivity of the contralateral ovary through suppression of pituitary follicle stimulating hormone release. Serum testosterone levels may be elevated if a significant theca cell component is present in the tumor (i.e., a granulosa-theca cell tumor, or GTCT). Testosterone is elevated in approximately 50 % of affected mares and is usually associated with stallion-like behavior. Progesterone concentrations in mares with a GCT are almost always below 1ng/ml, since the tumor does not produce progesterone and normal follicular development, ovulation, and corpus luteum formation generally do not occur.

Granulosa cell tumors are usually surgically removed if the tumor affects follicular development on the contralateral ovary, causes behavioral abnormalities, or is a source of colic. Surgical approaches for tumor removal include colpotomy, flank and ventral midline laparotomy, and laparoscopy. Ovulation from the remaining ovary will occur approximately six to eight months after tumor removal. Attempts at inducing follicular development and ovulation in the remaining ovary within one month after tumor removal by the administration of GnRH has not been successful.

Other ovarian tumors include cystadenoma (a tumor of the surface epithelium of the ovary) and teratoma or dysgerminoma (germ cell tumors). These tumors are not hormonally active and do not cause regression of the contralateral ovary.

Older maiden mare syndrome

An older maiden mare (i.e. ~13 to 15 years of age or older) may have a cervix that fails to relax during estrus. Breeding or insemination of a mare with a cervix that does not relax will result in accumulation of inflammatory fluid in the uterus and decreased pregnancy potential. A vaginal speculum examination and a digital (manual) examination of the cervix when the mare is in heat will determine the degree of cervical relaxation and the probability of uterine fluid accumulation after breeding. A reproductive management plan for an older maiden mare may include:

- The mare should be bred or inseminated only once, just prior to ovulation
- The uterus should be rinsed out or lavaged four to six hours after breeding
- Administration of one or more doses of oxytocin may also be helpful to promote uterine contractions and evacuation of uterine fluid
- An ultrasound examination should be performed the day after insemination to confirm that the mare ovulated and evaluate the uterus for fluid accumulation

- Topical administration of prostaglandin E_1 (Misoprostol; 1,000 to 2,000 µg) or N-Butylscopolammonium (Buscopan®) cream may be utilized to promote cervical relaxation

Persistent mating-induced endometritis

A transient inflammatory response in the endometrium is an inevitable consequence of mating by either natural service or by artificial insemination. Evidence indicates that most of the uterine inflammation is due to an antigenic response to the presence of spermatozoa.

Diagnosis is made on the basis of the presence of echogenic fluid in the uterine lumen on ultrasound examination 12 to 24 hours or more after insemination of the mare, as most normal mares will have cleared the inflammatory fluid associated with the insemination of spermatozoa within that time period. Additional diagnostic tests are not typically performed, but could include detection of the presence of white blood cells on cytology and an absence of bacterial growth on culture.

The inflammatory response is characterized by an influx of neutrophils into the uterine lumen and serves to clear the uterus of non-viable spermatozoa, seminal plasma and possible bacterial contamination. The inflammatory response begins within one-half hour after insemination. Neutrophil numbers are highest at approximately eight hours after mating and neutrophils disappear by 24 to 48 hours in normal mares. The intensity of the inflammatory response may be dependent on the concentration of spermatozoa introduced into the uterus. Insemination with frozen-thawed spermatozoa into the equine uterus may lead to an enhanced inflammatory response, due to the absence of seminal plasma in frozen semen.

A majority of young, reproductively normal mares are capable of eliminating the inflammation within 24 to 48 hours of breeding. In contrast, the post-mating inflammatory response may develop into a pathologic condition in older susceptible mares that cannot physically clear fluid and inflammatory products from their uterus. If the uterine inflammation persists, the embryo will not survive when it enters the uterus five to six days after ovulation. Persistent uterine inflammation may also result in premature luteolysis or short-cycling. Approximately 15 % of mares experience persistent endometritis after breeding by natural service or insemination.

Management of persistent mating-induced endometritis is aimed at limiting the severity and duration of the inflammatory response and clearing the uterus of fluid, inflammatory by-products and bacteria. The number of matings or inseminations should be limited in susceptible mares, and artificial insemination should be used to reduce the number of bacteria introduced into the uterus, if permitted by breed regulations. Oxytocin (20 IU) may be given intravenously four to eight hours after mating to stimulate uterine contractions and promote physical clearance of fluid and inflammatory by-products. The uterine lumen may be lavaged with one to four liters of sterile saline or sterile lactated Ringer's solution four to 24 hours after mating. The lavage is typically performed using approximately one liter of fluid at a time and is repeated until the effluent fluid appears clear. Intrauterine antibiotics are generally not necessary in mares with persistent mating induced endometritis, unless the presence of bacteria is suspected or has been confirmed.

Bacterial endometritis

Bacterial endometritis is a significant cause of decreased reproductive performance in mares due to failure of conception or early embryonic loss. A majority of young mares rapidly eliminate bacterial contamination of the uterus following mating, parturition, intrauterine manipulations or other events and are considered to be 'resistant' to infection. In contrast, some older mares may be unable to spontaneously eliminate pathogenic organisms from their uterus and are considered to be 'susceptible' to infection.

Factors predisposing mares to uterine infections include contamination at breeding, pooling of urine in the anterior vagina and uterus, trauma from parturition or breeding, and failure of natural uterine defense mechanisms. Poor perineal conformation, decreased muscular tone of the vulva and cranial displacement of the anal sphincter may lead to aspiration of air and fecal material into the reproductive tract.

Cytologic examination of an endometrial brush, swab or low volume lavage sample may reveal an increase in neutrophils (>5 neutrophils per high power field or more than one neutrophil per ten endometrial

epithelial cells) and, potentially, bacterial organisms. A uterine culture may detect the presence of a specific organism and antibiotic susceptibility tests can subsequently be determined. Polymerase chain reaction (PCR) analysis may be helpful in the detection of DNA from microbial organisms in uterine samples. An endometrial biopsy may be useful in predicting susceptibility, as mares with poor biopsy scores (i.e. Kenney grade III) are more likely to be susceptible to infection than mares with grade I biopsy scores.

The most common bacterial organisms cultured from mares with chronic endometritis are *Streptococcus equi* subsp. *zooepidemicus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and, in some cases, *Staphylococcus aureus*. The most common anaerobic organism cultured from the equine uterus is *Bacteroides fragilis*.

The general principles of treatment are to remove the source of infection, aid in physical clearance of the uterus, eliminate pathogenic organisms by local infusion or systemic administration of antimicrobial agents and reduce future contamination by enhanced reproductive management.

- Correction of any predisposing factor for infection
- Uterine lavage for removal of residual fluid, inflammatory by-products and bacteria
- Enhancement of uterine muscle contractility with oxytocin (20 IU, intravenously or intramuscularly) or prostaglandin (i.e. cloprostenol, 250 µg, intramuscularly)
- Intrauterine antibiotics (Table 4). Recent data suggest that dilution of antibiotics with Tris-EDTA or DMSO may be more effective in bacterial killing or elimination of bacterial biofilm than infusion of antibiotics alone
- Systemic antibiotic administration (Table 5)

Additional considerations

- Consider changing stallions if no pregnancy is obtained after three estrous cycles and all other diagnostic tests on the mare are normal.
- Consider infusion of kerosene for mares with chronic fluid (especially mucus) accumulation in the absence of infectious endometritis and/or cervical issues.
- Consider oviductal blockage as a potential cause of infertility if the mare has had a history of fertility, the stallion(s) utilized are fertile, the mare is bred with an adequate number of progressively motile, morphologically normal sperm, and all standard diagnostic tests are normal.

Suggested reading

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Table 1. Common and less common reproductive problems in mares.

Common Reproductive Problems	Less Common Reproductive Problems
Behavior Adverse behavior when in estrus Silent heat	**Behavior** Persistent estrus Stallion-like behavior
Ovary Anovulatory follicles Failure to ovulate after administration of hCG or a GnRH agonist Persistent corpus luteum Premature luteolysis (endometritis)	**Ovary** Ovarian tumors Failure of follicular development
Oviduct Parovarian cysts	**Oviduct** Oviductal blockage Salpingitis
Uterus Persistent mating-induced endometritis (PMIE) Bacterial endometritis Uterine cysts Endometriosis	**Uterus** Fungal endometritis Pyometra Persistent endometrial cups Tumor (i.e. leiomyoma) Foreign body
Cervix Failure of cervical relaxation	**Cervix** Cervical lacerations Cervical adhesions Tumor (i.e. leiomyoma)
Vagina/Vestibule Urovagina Vericose veins Imperforate hymen	**Vagina/Vestibule** Lacerations Adhesions Vaginitis
Perineum Poor conformation Inadequate vulva tone Perineal lacerations Melanoma (grey mares)	**Perineum** Squamous cell carcinoma Coital exanthema (EHV-3)
Miscellaneous Pituitary pars intermedia dysfunction (equine Cushing's disease)	**Miscellaneous** Chromosomal abnormalities Mastitis Inappropriate lactation

Table 2. Additional diagnostic tests that may be performed to identify reproductive issues in a problem mare.

Test	Indication
Laparoscopy	Direct visualization of the serosal surface of the ovary, oviduct and uterus and the abdominal cavity. Also used in ovarian biopsy, evaluation of oviductal patency and the application of prostaglandin E_2 to the oviductal surface
Hydrotubation of the oviducts	Performed by cannulation of the uterotubular junction via hysteroscopy in mares suspected of oviductal blockage
Oviductal flush	Performed by laparotomy or laparoscopy; used both diagnostically and therapeutically in suspected cases of oviductal blockage
Oviductal patency test	Deposition of fluorescent microbeads or starch granules within the infundibulum and subsequent examination of the uterine lumen for passage of the test material is used diagnostically to evaluate oviductal patency
Oviductal PGE_2 application	Direct application of prostaglandin E_2 can be used diagnostically and therapeutically in suspected cases of oviductal blockage
Ovarian Biopsy	Laparoscopic collection of an ovarian biopsy sample for histologic evaluation may be used in the diagnosis of ovarian pathology
Test breed	Breeding to a highly fertile stallion can be used diagnostically to help determine if the mare is a cause of subfertility or infertility

Table 3. Hormone concentrations in the normal non-pregnant mare and mares with a granulosa cell tumor.

Hormone	Normal Range	Levels in GCT mares
AMH	< 4.0 ng/ml	>4.0 ng/ml
Inhibin	0.1 to 0.7 ng/ml	>0.7 ng/ml
Testosterone	20 to 45 pg/ml	>45 ng/ml (if GTCT)
Progesterone - Estrus	<1.0 ng/ml	<1.0 ng/ml
Progesterone - Diestrus	>1.0 ng/ml	

Table 4. Dosages of antibiotics used for intrauterine infusion.

Medication	Dosage	Comments
Amikacin sulfate	1 to 2 grams; buffer with 10 to 20 mls sodium bicarbonate (8.4 %) then q.s. to 50 to 100 mls with sterile saline	Antibiotic (gram-negative spectrum)
Ampicillin	1 to 2 grams, reconstitute in 50 to 100 mls sterile saline	Antibiotic (gram-positive spectrum primarily)
Ceftiofur	1 gram, reconstitute with 20 to 60 mls sterile water	Antibiotic (broad spectrum)
Ciprofloxacin	400 mg, reconstitute in 50 mls sterile water	Antibiotic (gram-negative spectrum)
Gentamicin	1 to 2 grams; buffer with 10 to 20 mls of 8.4 % sodium bicarbonate; qs to 50 to 100 mls sterile saline	Antibiotic (gram-negative spectrum)
Potassium Penicillin	5 million units; reconstitute in 50 to 100 mls sterile saline	Antibiotic (gram-positive spectrum)
Procaine Penicillin	15 mls; dilute to 50 to 100 mls in sterile saline	Antibiotic (gram-positive spectrum)
Ticarcillin/Clavulanic acid	3.1 grams; reconstitute to 50 to 100 mls with sterile saline	Antibiotic combination; clavulanate blocks penicillinase; used for gram positive organisms and *Pseudomonas aeruginosa*

Table 5. Systemic antibiotics used in the treatment of bacterial endometritis.

Medication	Dosage	Route	Frequency
Ceftiofur crystalline free acid	6.6 mg/kg	IM	q 4 days
Ceftiofur sodium	1.1 to 2.2 mg/kg	IV or IM	q 12 h
Enrofloxacin	5 mg/kg (IV) 7.5 mg/kg (PO)	IV PO	q 24 h
Gentamicin	6.6 mg/kg	IV	q 24h
Penicillin (procaine)	22,000 IU/kg	IM	q 12h
Trimethoprim-sulfamethoxasole	30 mg/kg	PO	q 12h

Practical diagnostics and therapeutics in bacterial endometritis

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Abstract

Bacterial endometritis is an important cause of subfertility in the equine breeding industry. Diagnosis of all cases of bacterial endometritis can difficult in a clinical setting. Collection of an endometrial sample for microbial culture and cytology can easily be processed within a veterinary practice to allow for a rapid diagnosis allowing for appropriate treatment to be performed. There are many options in treating acute cases of bacterial endometritis. Recently, there have been developments in improved treatment strategies for bacteria residing in a biofilm with the combination of antibiotics and non-antibiotic therapies. To stimulate latent bacteria to enter a growth phase products such as bActivate or postbreeding inflammation can awaken these dormant bacteria. Obtaining an accurate diagnosis of bacterial endometritis can allow for appropriate treatment selection and resolution of bacterial endometritis.

Keywords: Mare, endometritis, bacteria, diagnostics, therapeutics

Introduction

Bacterial endometritis is common cause of reduced fertility in equine reproduction. Identification of the causative organism can help the practitioner develop an appropriate treatment plan. The goal of this review is to provide a clinical perspective on the diagnosis and treatment of bacterial endometritis.

Diagnosis of acute bacterial endometritis

Diagnosis of acute endometritis is usually performed via double-guarded uterine culture swab and cytology brush. This allows for detection, identification, and characterization of microbial organisms and evaluates the uterine lumen for the presence of polymorphic neutrophils (PMNs) indicate of active inflammation.

The initial database should consist of both a uterine culture and cytology sample. Individually, a uterine culture or cytology has a low sensitivity for the diagnosis of infectious endometritis.[3,5] However, when a positive culture or cytology sample is used to determine a positive case of infectious endometritis the sensitivity is improved to 0.42.[3] Depending on the causative infectious organism the cytology results may change, for example *Streptococcus zooepidemicus* is typically associated with a positive cytology sample as compared to *E. coli* which is typically associated with a cytology sample negative for inflammatory cells.[6] Additionally, either a positive culture or cytology should be considered diagnostic of infectious endometritis as these results are associated with reduced pregnancy rates.[5-7] Due to these findings it is suggested that the minimal database for evaluation of infectious endometritis be both a guarded swab for microbial culture and a guarded cytology brush for cytologic evaluation.

Cytology samples collected from a cotton swab are associated with increased cellular damage and decreased diagnostic capability.[1-4] The sensitivity of a swab for diagnosing inflammation in the uterus was also lower (0.00) as compared to a cytology brush (0.17).[3] The guarded cytology brush provides a sample with better cellularity and minimal artifacts from the collection process as compared to a sample collected from a swab.[1-4]

A limiting factor with all swab or brush collection protocols is the fact that the sample is obtained from a very small percentage of the endometrial surface or uterine lumen and the sample may not be representative of the entire uterus. A clinical tip to improve the diagnostic capability of the swab or brush is that if uterine fluid is detected on ultrasound examination prior to collection of the sample the swab and brush should be extremely wet with this fluid. If the swab/brush are relatively dry the sample may not have been in the fluid and a second sample should be collected.

Small volume lavage

A low volume lavage (LVL) may be performed in 'problem' mares to provide a more complete sampling of the entire uterine lumen.[8,9] A volume of 150 mL of sterile saline or lactated Ringer's solution (LRS) is infused into the uterus by gravity flow. The fluid is gently massaged throughout the uterine lumen by palpation per rectum. The infused fluid is recovered by gravity flow after lowering the original fluid bag to ground level. Recovery is facilitated by transrectal massage of the uterus and intravenous administration of 20 units of oxytocin. Uterine effluent fluid is transferred into one or two sterile 50-ml conical tubes and centrifuged at 600 x g for 10 minutes. The supernatant is aspirated or decanted, and the pellet can be submitted for microbiology culture, picked up with a cotton tipped swab and gently rolled onto a glass slide for cytology evaluation. There is often an increase in cellular artifacts (damaged cells, disrupted cells, etc.) associated with the centrifugation process versus samples collected by uterine swab or brush. The fluid and processing techniques associated with a LVL sample had no effect on the quality or diagnostic capabilities of the resulting cytology smear.[1]

The use of a LVL for the diagnosis of infectious endometritis results in an improved sensitivity for culture of 0.71 and cytology of 0.80. The improved sensitivity with this technique results in less false negative samples as compared to a guarded swab or brush cytology sample. However, increased time and personnel required to perform a LVL has prevented this diagnostic technique from replacing a traditional guarded culture and cytology same as the initial diagnostic technique to screen for infectious endometritis.

Evaluation of samples

Microbiology

For many equine reproduction practitioners, microbiology can be frustrating. By the time a culture is taken, sent to a laboratory and the results reported, the mare has already ovulated and the window of time for directed intervention has passed for that cycle. Likewise, the prospect of performing in-house microbiology can seem overwhelming, time consuming and tedious. The reality is that in-house microbiology can be practical, simple, and inexpensive.

Samples should be applied to agar that is capable of supporting growth of common equine uterine pathogens.[10,11] Common agar that has been utilized for cultivating equine uterine bacterial pathogens are trypticase soy agar (TSA) with 5% sheep blood, MacConkey agar, and chromogenic agar.

In our clinical program, we utilize a quad plate consisting of TSA, MacConkey, gram-positive chromogenic and gram-negative chromogenic agars. The swab or pellet from a LVL should be applied directly to the agar surface and streaked back and forth over approximately 30% of the agar surface (primary streak) a flamed inoculation loop or sterile swab is passed through the primary streak once and continue to streak the remainder of the plate (secondary streak). The whole idea is to dilute or spread out the colonies so that individual colonies will be available to work with and identify. This allows for the amount of growth to be characterized and the resulting severity of the infection predicted (Table 1).

Initial results are available after 12-24 hours of cultivation but plates are monitored for 72 hours for the presence of slow growing bacterial organisms. In our clinical setting this allows for the results of the microbiology culture to be known by 7:00 AM when mares start to be examined, allowing for these results to be considered when trying to optimize breeding management.

Common bacterial isolates can be easily identified by their growth characteristics on common agar (Table 2 and 3). Recent work in our laboratory showed that uterine isolates of *Streptococcus zooepidemicus, E. coli, P. aeruginosa* and *K. pneumoniae* cultivated on TSA with 5% sheep blood, MacConkey agar and a gram positive/negative selective chromogenic agar were easily identified by untrained personnel based on comparison to common bacteria isolated from the equine uterus. Identification based on growth characteristics was 96% accurate as compared to two commercial identification systems (38% and 86%).

Antibiotic sensitivity (Kirby-Bauer method) can be done easily by selecting single colonies from above and streaking the bacteria onto Mueller Hinton agar and applying antibiotic discs directly to the

agar surface. After 18-24 hours of cultivation the zone of bacterial inhibition can be measured and susceptibility or resistance determined for that particular antibiotic. This method allows the practitioner to screen for the common antibiotics that they use in clinical practice. Additional information including color images of common culture plates and techniques in clinical practice can be found at erl.colostate.edu.

Cytology

Endometrial cytology is a rapid and inexpensive technique to detect the presence of endometritis. A diagnosis can often be made based on cytology results alone and suitable therapy initiated several days prior to obtaining results of microbial culture.

The glass slides with the sample applied from the cytology brush, LVL pellet, or uterine biopsy are allowed to air dry and stained with a modified Wright's stain. If a prolonged period of time (>12 hours) is anticipated between collection and staining, a methanol or spray fixative can be used to maintain cellular architecture. Additional stains that may be considered are a Gram stain or a fungus specific stain such as Grocott Methenamine Silver stain.

Evaluation of uterine cytology slides should be thorough and systematic. An initial evaluation is performed at low magnification to determine if there is adequate cellularity to provide accurate interpretation. If the slide is determined to be of inadequate cellularity (i.e. a low number of uterine epithelial cells [UEC]) to be of diagnostic quality, a new sample should be collected.

If the sample is determined to be of diagnostic quality, the slide is then evaluated at 400x (10x eyepiece x 40x objective) magnification with multiple high power fields examined for UECs (individual cells and rafts of cells), white blood cells, debris, red blood cells, bacteria, yeast, fungal organisms, and spermatozoa. Evaluation of a field of view at 400x magnification is often referred to as a "high power field" or "hpf". Evaluation at 1000x (10x eyepiece x 100x oil-immersion objective) magnification may be required to confirm the presence of bacterial and fungal pathogens detected at 400x. A check sheet can be utilized to rapidly and accurately record the observations.

Uterine epithelial cells. Uterine epithelial cells can range from cuboidal in anestrus to tall columnar during the estrous cycle.[12] A majority of UECs are not ciliated, but ciliated cells are often observed in cytology preparations. A range of UECs may be noted including large rafts of cells, intact individual cells, or disrupted UECs. Squamous epithelial cells are rare and usually only present in postpartum mares or mares refluxing urine into the uterus.[13-15] Squamous epithelial cells are typically evidence of cervical cell contamination. A large number of disrupted or degenerate uterine epithelial cells may indicate improper sample preparation, handling or storage prior to staining or may be associated with a chronic uterine infection.

Debris. Debris may be classified as none/minimal (< 25% of the slide), mild (25-50% of the slide), or moderate/severe (>75% of the slide). The presence of moderate to severe debris has been associated with bacterial endometritis. Additional diagnostic tests such as a LVL may be indicated if a traditional uterine cytology sample shows moderate or severe debris with minimal evidence of inflammation (i.e. a low number of white blood cells [WBCs]).

White blood cells. Neutrophils are the predominant WBC identified on uterine cytologic preparations. Neutrophils are 10-12 μm in diameter (~twice as large as a red blood cell [RBC]), with a single nucleus that may be indented or divided into three to five lobes or segments. A cytology sample collected from a normal mare in estrus should have very few or no neutrophils; occasionally a rare neutrophil may be noted associated with blood contamination during the collection process. Neutrophils will be present in the uterine lumen following breeding , after uterine lavage or infusion, during the postpartum period or in cases of endometritis.[12]

Other white blood cells such as macrophages, lymphocytes or eosinophils are not commonly found on equine uterine cytology preparations. Lymphocytes are approximately 7 μm in diameter (same size as a RBC), are round to oval, and have only a small amount of cytoplasm (Figure 8). Macrophages are approximately 20 μm in diameter with abundant blue staining cytoplasm filled with various sized vacuoles. Lymphocytes and macrophages are found in the postpartum mare and in chronic uterine

infections. Eosinophils are 12-15 µm in diameter, with blue staining cytoplasm that contains multiple pink or red granules.

The average the number of WBCs per hpf is determined after evaluating at least ten hpf in multiple areas of the slide. The following categories may be used to define the presence/absence of white blood cells: normal (no WBC to rare WBC/hpf), mild inflammation (1-2 WBC/hpf), moderate inflammation (3-5 WBC/hpf) and severe inflammation (>5 WBC/hpf) (Table 4).[6,16,17] Another method used to categorize the inflammatory response is the number of WBCs per uterine epithelial cell. A ratio of 1 WBC to 20-40 epithelial cells has been used as a gauge of the degree of inflammation.[8,13] If the sample is of adequate cellularity there is little to no difference between the two techniques in the ability to categorize the degree of inflammation. The number of WBC per hpf does not apply to samples prepared from LVL as epithelial cells and white blood cells are concentrated during centrifugation. However, a ratio of WBC to UEC would still be appropriate.

Categorizing the degree of inflammation represented in a cytological sample from a LVL can be difficult. Centrifugation of the uterine effluent concentrates uterine epithelial cells, white blood cells, microbial organisms and debris into a pellet. The pellet is subsequently smeared onto a glass slide, stained and evaluated. A normal mare should have very few or no WBCs noted in the cytology from a LVL. Mares with mild uterine inflammation often have >5-10 neutrophils per hpf, whereas mares with more severe inflammation usually have >10 neutrophils per hpf. The presence of microbial organisms should be interpreted with caution, as there is a higher risk potential for contamination during sample collection with a LVL procedure versus use of a double guarded uterine swab or brush.

Red blood cells. Red blood cells are 6 µm in diameter with a central pallor. These cells are commonly found in low numbers (i.e. <4/hpf) on routine cytologic evaluation. Excessive numbers of red blood cells may indicate irritation to the endometrium from infection, infused compounds or aggressive sampling technique.

Bacteria. Bacteria can be visualized on a uterine cytology sample but often require 1000x (10x eye piece x 100x oil-immersion objective) magnification to differentiate them from debris. Presence of bacteria engulfed within white blood cells can help determine if the bacteria are due to infectious endometritis or contamination. The four most common bacterial pathogens of the equine uterus are *Streptococcus equi* subsp. *zooepidemicus*, *Escherichia coli*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*. *Strep. equi* subsp. *zooepidemicus* is a gram positive coccus approximately 1.0 um in diameter that form chains of various length. *E. coli*, *K. pneumonia*, and *P. aeruginosa* are gram negative rods varying in size from 3 to 6 µm in length and cannot be differentiated accurately based on cytologic evaluation.

The first step in interpretation of an endometrial cytology sample is determination if the sample is of adequate quality to be diagnostic. Subsequently, the sample is evaluated for evidence of inflammation and other abnormalities. The presence of more than 1 neutrophil per hpf from a traditional swab or brush cytology sample is an indication of inflammation, but does not necessarily indicate infection with a pathogenic organism. Typically, the number of neutrophils per hpf increases as the degree of inflammation increases within the uterus. In severe cases of endometritis neutrophils may be so prevalent that few other cells can be identified. For an in-depth review of endometrial cytology with color photos please see Ferris et al.[2]

Summary of diagnostics for bacterial endometritis

Detection of infectious endometritis while challenging in certain cases usually can be easily diagnosed or rule out using a sample from a guarded swab and cytology brush, LVL, or uterine biopsy. These samples are routinely submitted for microbial culture and cytological evaluation. However, there is not a gold standard diagnostic sample or analysis of the sample that is quick to perform, inexpensive, and returns rapid results.

In a clinical setting an option for screening mares with infections endometritis is to use a guarded culture swab and cytology brush for microbial culture and cytological evaluation. If the results are positive the causative organism and antibiotic susceptibility can be determined to generate an appropriate

therapeutic plan. If the results are negative for infectious endometritis but the mare has several risk factors or clinical signs (intra-uterine fluid, cervical discharge, etc) further diagnostics are warranted such as a LVL or uterine biopsy to confirm or rule out infectious endometritis.

Treatment of bacterial endometritis

Mares diagnosed with bacterial endometritis can be divided into those with acute and chronic infections. While these terms have multiple meanings, for this discussion acute endometritis will describe cases in which mares are exposed to bacterial pathogens and an infection develops. The term chronic endometritis is going to be used in cases where acute bacterial endometritis is detected, appropriate therapy initiated, yet the infection continues persist despite treatment.

Acute bacterial endometritis

If the culture is from one of the bacterial genus known to be pathogenic (*Streptococcus equi* subsp. *zooepidemicus, Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae)* in the equine uterus treatment is always warranted. However, there is a long list of bacteria with questionable pathogenicity, these organisms may be associated with an infection when in heavy growth, and with evidence of clinical disease (positive cytology, intrauterine fluid, or history of short cycling; Table 2).[18,19] The therapeutic plan is to aid the uterus in clearing infectious agents and inflammatory debris.

Intrauterine therapy. For mares with large volumes of intrauterine fluid a uterine lavage will be performed prior to antibiotic treatment. The goal of the lavage with 0.9% saline or LRS is to reduce the number of infectious organisms, remove inflammatory debris from the uterus and potentially enhance luminal defense mechanisms by inducing local irritation and influx of PMNs into the uterine lumen. We will typically treat these mares with three days of intrauterine therapy based on antimicrobial susceptibility patterns (Table 5). Ceftiofur sodium is our antibiotic of choice for empirical therapy, if therapy is initiated before antimicrobial susceptibility is returned.

Ecbolics such as oxytocin (5 to 20 IU administered IM or IV) or cloprostenol (250 µg IM) should not be given during the lavage or shortly after the antibiotic infusion as this may cause the majority of the antibiotic to be immediately evacuated from the uterus. We try to collect our culture and cytology samples in early estrus so that if bacterial endometritis is detected therapy can be initiated and the mare still bred on the same estrous cycle.

Part of the treatment plan needs to address issues with perineal conformation with procedures such as Caslick's surgery, perineal body reconstruction, or cervical laceration. Any mare with less than two-thirds of the vulva below the pelvic brim or >10 degree of angulation of the vulva warrants Caslick's surgery. Severe cases with minimal to no vulva below the pelvic brim or >60° of angulation to the vulva warrant perineal body reconstruction. Following treatment, an ultrasound examination should be performed as the mare comes back into estrus for the presence of intrauterine fluid and confirming the uterine environment is free of infection by uterine culture and cytology during the next estrus.

Systemic therapy. An additional consideration is that in many of these mares a 60 ml infusion of antibiotics results in several hundred mLs of fluid in the uterine lumen the next day. For these mares we will often switch to systemic antibiotics if appropriate based on antimicrobial susceptibility (Table 6). Additionally, if the culture was not collected early in estrus the results may be obtained on the day of or the day after ovulation. Instead of intrauterine therapy inflaming the uterine environment systemic therapy may be more appropriate. A common antibiotic to use systemically is ceftiofur crystalline free acid (Excede®, Zoetis, Parsippony, NJ) to provide 10 days of therapy.

Chronic bacterial endometritis

Chronic cases of bacterial endometritis are those that have been treated traditionally as described for acute infections and are refractory to treatment. Current explanations as to why these cases are refractory to treatment are bacteria are protected by a biofilm from antibiotic exposure; antimicrobial resistance develops during treatment; or the mare become re-infected with the same genus of bacteria.

Due to these issues, the management of mares with chronic infections is often much more intensive from a therapeutic perspective.

While clients want to start treating these infections quickly, it is advantageous when developing a treatment plans should be focused on helping kill bacteria, disrupt a biofilm and remove debris from the uterine lumen. The treatment plan will start with a uterine lavage as described in acute endometritis. Through a series of in vitro studies conducted to assess biofilm dispersal and/or bacterial killing for antibiotics and non-antibiotic agents alone or in combination against gram-negative bacteria. Results indicate that antibiotics and non-antibiotic agents are more effective against biofilm if administered concurrently (i.e. in the same syringe). Clinical treatments can easily be prepared for local infusion into the uterus based on the in vitro data (Table 7). The amount of either antibiotic or non-antibiotic agent for each infusion are the minimum effective concentrations against *E. coli, K. pneumoniae* and *P. aeruginosa*. The treatment period should be at least 72 hours in duration, with repeated treatments every 24 hours (i.e. a uterine infusion of the selected combination once every 24 hours for three consecutive days). This treatment protocol resulted in complete biofilm dispersal and bacterial killing in vitro.

It is important to note that some non-antibiotic agents and antibiotics should not be combined in the same syringe. For example, the in vitro data indicated that mixing acetylcysteine with antibiotics in the same syringe resulted in reduced activity of the antibiotics.

We recommend antibiotic sensitivity testing for all gram-negative organisms. Bacteria inherently resistant to an antibiotic will still be resistant when that antibiotic is used in combination with a non-antibiotic agent.

Latent bacteria

The goal for treating mares with latent or dormant bacteria is to get the bacteria to move from the dormant state into a metabolically active state in which identification and treatment can be performed. Recent work by Petersen et al has shown that dormant *Streptococcus zooepidemicus* can be activated by infusing a proprietary media (bActivate) into the uterus.[20] After the infusion (24 hours) 64% (15/25) mares were culture positive for *Streptococcus zooepidemicus* as compared to 8% (1/12) mares infused with phosphate buffered saline.[20] The proprietary medium is capable of getting bacteria to convert from the dormant state to a metabolically active state and treatment can be initiated.

Interestingly, it should be noted that breeding also may result in bacteria being activated from a dormant state. Recent work by Christoffersen et al showed that 55% (16 of 29) of mares with a negative culture prior to breeding but retained fluid after breeding were positive for growth of *Streptococcus zooepidemicus*. Christoffersen et al concluded that it was more likely to be dormant *Streptococcus zooepidemicus* that was reactivated as compared to introduction at the time of breeding.[21] The development of intrauterine fluid after breeding in barren mares could be due to both inflammation from breeding but also reactivation of dormant bacteria.

Further work is warranted to determine the incidence of latent bacteria in the equine breeding industry and to determine the best method for activating the dormant bacteria. Latent bacteria should be a differential for sub-fertility in barren mares or in mares that have unexpected fluid after breeding.

Conclusion

Diagnosis of bacterial endometritis is required in developing an appropriate treatment plan. Microbial culture and cytology samples can easily be evaluated in a clinical practice allowing for faster return of results. There are many options for the treatment of bacterial endometritis especially recent work in chronic cases involving biofilm associated infections or latent bacteria.

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Table 1. Determination of bacterial growth characteristic relative to the number of colonies of each organism present at 24 and 48 hours.

Growth	Number of Colonies
No growth	0
Very light	≤ 2, primary streak
Light	3 – 5, primary streak
Moderate	> 5, into secondary streak
Heavy	> 5, no single colonies

Table 2. Common bacteria cultured from the equine uterus.

Etiological Agents Detected in the Equine Uterus	
Bacteria known to be pathogenic	
Streptococcus sp. -β-hemolytic -*equi* ssp. *zooepidemicus* *Escherichia coli* *Pseudomonas aeruginosa* *Klebsiella pneumoniae*	
Bacteria with questionable pathogenicity	
Aerobic Bacteria Methicillin resistant Staphlococcus aureus Streptococcus sp. -α-hemolytic -non-hemolytic -faecalis -equisimilis Bordetella bronchiseptica Proteus sp -mirabilis -vulgaris Staphalococcus sp. -aureus -albus -intermedius Serratia sp. Corynebacterium sp.	Citrobacter sp. Enterobacter sp. Bacillus sp. Actinetobacter sp. Micrococcus sp. Pasturella sp. **Anaerobic Bacteria** Bacteroides fragilis Fusobacterium sp. Clostridium sp. -perfringens -difficile Mycoplasma spp. Chlamydia spp.

Table 3. Bacterial growth characteristics for deification of common equine uterine pathogens. Characteristics of chromogenic agar may only specific to Spectrum Agar, VetLabs.

Organism	Gram Stain	Morph	Culture Characteristics				Comments
			TSA-5% Sheep Blood	MacConkey	Gram + Chromagar	Gram - Chromagar	
Streptococcus zooepidemicus	Pos	Cocci, chains	Small, white, round colonies with beta hemolysis (0.5-1mm)	No Growth	Small, light blue colonies	No Growth	Training is required to differentiate alpha and beta hemolysis
Escherichia coli	Neg	Rods	Cream to gray colored colonies +/- alpha hemolysis (2-3 mm)	Medium sized, grey to pink colonies	No growth	Medium, pink to red colonies	
Klebsiella pneumoniae	Neg	Rods	Large grey mucoid colonies (2-4 mm)	Large, pink mucoid colonies	No Growth	Large, dark blue colonies with slight pink halo	Pink halo may take 24-36 hours to develop and is faint
Pseudomonas aeruginosa	Neg	Rod	Flat metallic blue colonies (3-4 mm)	Large, pale greenish colonies	No Growth	Transparent white to green colonies	"Grape-like" odor on blood agar, fluorescence with UV light
Staphylococcus aureus	Pos	Cocci, clusters	Medium, cream to gold colonies, +/- beta hemolysis (2-3 mm)	No growth or limited growth of pink colonies	White to yellow colonies	No Growth	

Table 4. Description of the number of neutrophils per hpf with corresponding inflammation classification for samples collected from a brush/swab.

Number of Neutrophils per hpf	Classification
0-Rare	Normal
1-2	Mild Inflammation
3-5	Moderate Inflammation
>5	Severe Inflammation

Table 5. Common antibiotics for intrauterine therapy in equine reproduction.

Medication	Dosage, Route, Frequency	Indications
Amikacin sulfate (Amiglyde-V®) (250 mg/ml)	1-2 grams; buffer with 10 to 20 mls sodium bicarbonate (8.4 %) then qs to 60 mls with sterile saline	Antibiotic (gram negative spectrum)
Ampicillin (1 gm vial)	1-2 grams, reconstitute in 60 mls sterile saline	Antibiotic (gram positive spectrum primarily)
Ceftiofur (Naxcel®)	1 gram, reconstitute with 20 to 60 mls sterile water	Antibiotic (broad spectrum)
Gentamicin (100 mg/ml)	1-2 grams; buffer with 10 to 20 mls of 8.4 % sodium bicarbonate	Antibiotic (gram negative spectrum)
Penicillin (Potassium) (5 million units/vial)	5 million units, reconstitute in 60 mls sterile saline	Antibiotic (gram positive spectrum)
Penicillin (Procaine) (300,000 units per ml)	15 mls, dilute to 60 mls in sterile saline	Antibiotic (gram positive spectrum)
Ticarcillin/Clavulanic acid (Timentin®) (3.1 gm per vial)	3.1 grams, reconstitute to 60 mls with sterile saline	Antibiotic combination; clavulanate blocks penicillinase; used for gram positive organisms and Pseudomonas aeruginosa

Table 6. Common systemic antibiotics used in equine reproduction.

Medication	Dosage, Route, Frequency	Indications
Ceftiofur sodium (Naxcel®) (50 mg/ml)	1.1 to 2.2 mg/kg, IV or IM, q 12h	Antibiotic (broad spectrum); used in equine reproduction for treatment of bacterial endometritis
Ceftiofur crystalline free acid (Excede®) (200 mg/ml)	3.0 mg ceftiofur equivalents/kg, IM; retreat in 4 days if needed	Antibiotic (broad spectrum); used in equine reproduction for systemic treatment of bacterial endometritis
Enrofloxacin (Baytril®) (50 or 100 mg/ml)	5 mg/kg, IV, q 24 hours or 7.5 mg/kg, PO, q 24h	Antibiotic (broad spectrum); used in equine reproduction for treatment of bacterial endometritis, specifically for resistant Pseudomonas sp.; Note: intra-uterine therapy of the commercial product is associated with severe necrosis and is not recommended

Table 7. Antibiotic and non-antibiotic combinations for the treatment of biofilm associated bacterial endometritis in mares

Tris EDTA- final concentration in the syringe should be 50 mM Tris and 3.5 mM EDTA
Note: Tris-EDTA and Tricide are similar; however Tricide is not equivalent to Tris-EDTA in regards to bacterial killing
To make Tris-EDTA: 16oz bottle of Dechra Triz-EDTA crystals; add 8 oz of sterile water (this is different than the bottle instructions).
The 2x concentration of Tris-EDTA solution will be further diluted by the antibiotics below to the proper final concentration.

Antibiotic	Drug Amount	Tris EDTA	QS	Final volume	Notes:
Amikacin (250 mg/ml)	4 mls (1 gram)	30 mls	16 mls sterile fluid (Saline, LRS, Sterile H2O)	60 mls	10 mls of 8.4% sodium bicarbonate should be added to the amikacin
Ceftiofur (1 gram reconstituted in 20 mls)	20 mls (1 gram)	30 mls	10 mls sterile fluid (Sterile H2O)	60 mls	
Ciprofloxacin (10 mg/ml)	40 mls (400 mg)	40 mls	0	80 mls	Split between two syringes

H2O2- 1% final concentration in the syringe
A 3% stock solution is available at many drug stores and veterinary distributors

Antibiotic	Drug Amount	H2O2	QS	Final volume	Notes:
Amikacin (250 mg/ml)	4 mls (1 gram)	20 mls	26 mls sterile fluid (Saline, LRS, Sterile H2O)	60 mls	10 mls of 8.4% sodium bicarbonate should be added to the amikacin
Ciprofloxacin (10 mg/ml)	40 mls (400 mg)	20 mls	0	60 mls	

DMSO- 30% final concentration in the syringe
99% stock solution is used for calculations below

Antibiotic	Drug Amount	DMSO	QS	Final volume	Notes:
Ceftiofur (1 gram reconstituted in 20 mls)	20 mls (1 gram)	20 mls	20 mls sterile fluid (Sterile H2O)	60 mls	
Ciprofloxacin (10 mg/ml)	40 mls (400 mg)	20 mls	0	60 mls	

Equine embryo transfer: clinical perspectives

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Abstract

Collection and transfer of embryos is common in equine veterinary practice. Keys to success are attention to detail, optimal reproductive management of donor mares, careful selection and management of recipient mares, adherence to guidelines for embryo recovery, evaluation and handling, plus a gentle transcervical transfer technique.

Key Words: Equine, embryo transfer, pregnancy

Introduction

Embryo transfer is a common technique in broodmare practice. The goal of this review is to provide a practical clinical perspective to various aspects of equine embryo transfer.

Embryo collection

Collection days

Equine embryos enter the uterus through the utero-tubular junction (UTJ) between 5.5 and 6 days after ovulation, at which time most equine embryos will be at morula or early blastocyst stage of development. Embryo recovery attempts in clinical practice are usually performed 6.5 to 9 days after ovulation. A collection attempt may be performed on day 6.5 or early on day 7 to procure a small (i.e. < 300 μm) embryo for cryopreservation.

A majority of mares in the United States are flushed on day 7.5 or 8 after ovulation because embryo recovery rates are high and a majority of embryos are blastocysts or expanded blastocysts and are easily observed under the microscope. The collection procedure is often delayed by one-half day for mares bred with frozen semen because of a slight delay in embryonic development (Table 1). In some cases collection is also delayed for older mares and mares bred after ovulation, although the data do not support either situation as routinely resulting in recovery of smaller embryos.

Equine embryos approximately double in size from day 7 to day 8 (Table 2) and double again between day 8 and day 9. Consequently, embryo collection on day 9 after ovulation usually results in the recovery of large embryos (i.e. > 1 to 2 mm in diameter).

Flush media

Options for flush media include a variety of commercially available 'complete' flush media that contain a Zwitterion-based buffer system, antibiotics and purified albumen or polyvinyl alcohol (PVA) as a surfactant or lactated Ringer's solution (LRS) or Hartmann's solution without additives. A recent study at CSU showed no difference in either embryo recovery rate or pregnancy rate after transfer using a complete flush medium or Hartmann's solution (Figure).

Embryos adhering to search dish

One potential advantage of a complete flush solution containing a surfactant versus a crystalloid solution devoid of surfactant is the perception that embryos may stick to the catheter, tubing, cup or Petri dish if flush fluids without a surfactant are used. Of the first 21 embryos recovered in 2017 using Hartmann's solution without a surfactant, 11 embryos adhered to the search Petri dish. All 11 embryos were gently 'unstuck' from the original search dish, washed in a commercial embryo holding medium containing polyvinyl alcohol (PVA) and transferred into a recipient mare. Nine of the 11 embryos (81.8 %) transferred resulted in a pregnancy. By comparison, 7 of the 10 embryos (70 %) that did not adhere to the original Petri dish resulted in a pregnancy after transfer.

Due to the high proportion of embryos adhering to the Petri dish when flushed with Hartmann's solution, a trial was performed evaluating embryo adherence to a variety of commercial plastic search dishes. It was noted that equine embryos tended to stick to dishes from some manufacturers and not to others. Consequently, our clinical embryo transfer program now utilizes a plastic search dish that is not associated with embryo adherence.

Extra flush procedure

If an embryo is not recovered following an initial series of three uterine lavages, one to two additional liters of medium are immediately infused into the uterus and 20 units of oxytocin is administered intravenously. The medium is allowed to remain in the mare for three minutes before being recovered by gravity flow aided by uterine massage per rectum. In a recent retrospective study of 208 embryo flush attempts, an embryo was collected during the first round of three lavages on 89 occasions (42.8 %). An embryo was collected on 30 're-flush' attempts, yielding a total of 119 positive flushes in 208 attempts, for a 57.2 % overall embryo collection rate.

Next-day re-flush

In rare circumstances a mare may be re-flushed the day after an initial negative embryo collection attempt. Usually this would be a mare that had a great cycle, semen was of high quality, the mare ovulated on schedule, no post-mating fluid accumulation was noted, and yet no embryo was recovered. In addition, a 'next-day re-flush' may be performed if that was the only estrous cycle in which the mare was bred that year. In a retrospective study at CSU, three embryos were recovered during a total of 31 'next-day reflushes' (9.7 %). In general, the recovered fluid is slightly cloudy and mild to moderate debris is present in the search dish on 'next-day re-flushes'.

Unfertilized oocytes

An unfertilized oocyte (UFO) is occasionally recovered during an embryo collection attempt. Since prostaglandin E_2 is required for embryo transport through the isthmus and utero-tubular junction, a UFO is usually only recovered if it passively follows a viable embryo. Two UFOs were recovered in a retrospective study of 208 flush attempts; in both instances an embryo was also recovered.

Embryo evaluation

Assessment of embryo grade, determination of developmental stage and measurement of embryo size are key components of an embryo evaluation. Accurate evaluation of embryos is important in a clinical embryo transfer program. For example, recovery of a small morula stage embryo from a Day-8 donor may dictate transfer into a Day-5 recipient mare that is synchronized more on embryo stage than on ovulation date. Another example is good cryopreservation success with embryos ≤ 300 µm in diameter and progressively less success with embryos significantly larger than 300 µm. Photographing embryos has become routine in many practices and the photograph is part of the donor mare's medical record.

Recipient management

Acquisition of quality recipient mares remains one of the most important components of a successful equine embryo transfer program. The best advice is to be very careful and selective when acquiring recipients, only maintain good quality recipients and find alternative careers for recipient mares that do not fit into an embryo transfer program.

Culture and cytology

Collection of samples for uterine culture and cytology is an important part of the evaluation process for recipient mares. Samples for culture and cytology may also be collected at other points throughout the breeding season for recipient mares that do not become pregnant after transfer or mares that have echogenic fluid in their uterus on ultrasound examination. It is estimated that 5 to 10 % of potential recipient mares have light to moderate growth of a potentially pathogenic bacterium or a

positive cytology or both. Once identified, affected mares should be treated and recultured before ever receiving an embryo. Mares that continue to have a positive culture should be culled.

Hormone treatment

Recipient mares are 'put under lights' beginning on December 1 to advance the first ovulation of the year. Unfortunately, it is not always possible to have a sufficient number of recipients with natural ovulations in the first month or two of the breeding season for all of the embryos that need to be transferred. Consequently, hormonal management of recipient mares is a necessity. In 2016, a total of 40 embryos were transferred into deep anestrus or transitional mares that had been treated with 6.6 mg estradiol 17β for two consecutive days followed by 5 to 7 days of a short-acting progesterone preparation (200 mg, IM, q 24h). Over the same time period 65 embryos were transferred into recipient mares with natural ovulations. Pregnancy rates at day 14 were 85 % and 75 %, respectively ($p>0.05$).

Long-acting progesterone preparations (i.e. 1,500 mg, IM, once per week) may be considered after a recipient mare is determined to be pregnant. Administration of a long-acting progesterone preparation at the time of transfer is discouraged as mares that do not become pregnant may take a long time to return to estrus.

Donor-recipient synchrony

Historically, the range of synchrony between a donor and a recipient was considered to be +1 (recipient ovulated one day ahead of the donor) to -2 (recipient ovulated 2 days behind the donor). Recent work at several ET facilities around the world has expanded the range to -3 and occasionally -4. For example, in 2016, a total of 14 embryos were transferred into recipient mares that had ovulated 3 days behind the donor and 12 of those recipient mares became pregnant (85.7 %).

Recipient size

Although research has shown that a recipient mare can carry a pregnancy from a much larger donor mare to term and give birth to a healthy foal, clinical experience has indicated that owners expect that a recipient mare should be approximately the same size as the donor.

Progesterone concentrations in recipient mares

It is usually assumed that after an ovulation is detected, a normal corpus luteum will form and that adequate progesterone will subsequently be produced. An early study at CSU showed that 3.7% (9 of 242) of estrous cycles in recipient mares were associated with progesterone values of < 4.0 ng/ml at 5 days after ovulation. In 2016, progesterone concentrations were determined 5 days after ovulation during 218 estrous cycles in recipient mares. The mean progesterone value 5 days after ovulation was 9.4 ± 3.7 ng/ml. A progesterone concentration of < 4.0 ng/ml was noted in 5.5 % of mares and a progesterone concentration of < 1.0 ng/ml was detected in 0.9% of mares (2 of 218). In the latter two cases, a corpus luteum apparently never formed after ovulation.

'Two-strike rule'

In the CSU embryo transfer program, a recipient mare is usually not utilized again if she does not become pregnant after having received two Grade 1 or Grade 2 embryos within a single breeding season. Historical data have indicated that pregnancy rates are low following transfer of a third embryo within the same season.

The 5-day check

Recipient mares should be examined 5 days after ovulation to determine if they qualify to receive an embryo on that cycle. Mares that are graded as 'acceptable' on this examination are available for use as recipients for the next 3 to 4 days. Criteria for evaluation of a mare on the 5-day check include:

- Quality of estrous cycle (follicle growth pattern, edema pattern, ovulation detection)
- Presence and ultrasonographic quality of the corpus luteum

- Progesterone level (if available)
- Tone of the uterus
- Tone of the cervix
- Absence of uterine edema
- Size of the recipient relative to size of the donor mare
- General physical health
- Behavioral characteristics
- Absence of reproductive abnormalities, medical issues or behavioral concerns

Factors that disqualify or decrease the likelihood of using an individual recipient mare include:

- Poor quality cycle
- Ovulation within 2 days after receiving prostaglandin
- Ovulation of an abnormally small follicle
- Failure of ovulation or development of a hemorrhagic anovulatory follicle
- Absence of uterine edema during the cycle
- Presence of echogenic fluid within the uterine lumen during estrus
- Presence of fluid within the uterine lumen during diestrus
- Positive uterine culture or positive cytology
- Presence of a significant medical condition or behavioral issue

Transfer of embryos

Transfer of an equine embryo into a recipient is where the science and art of embryo transfer merge together. There are numerous minor variations on the general theme of how to transfer an embryo. A few clinical suggestions include:

- Always ultrasound the recipient mare immediately prior to transfer
- Lightly sedate the recipient mare (i.e. acepromazine, 20 mg, i.v.)
- Administer a non-steroidal anti-inflammatory drug prior to transfer (i.e. 500 mg flunixin meglumine, i.v.)
- Utilize a chemise to protect the transfer instrument from contamination
- Gently pass the transfer gun or pipette through the cervix without inserting a finger into or through the cervix
- Check the position of the instrument tip by palpation per rectum
- Gently deposit the embryo while slowly withdrawing the transfer instrument
- Consider using a Cassou gun and 0.25 ml straw for embryos $\leq$ 1,000 μm; use an insemination pipette for embryos > 1,000 μm
- Always rinse out the tip of a Cassou gun into a Petri dish after transfer to make sure that the embryo was not retained in the instrument

Management of the recipient mare after transfer

Minimize stress

Minimizing stress in the recipient mare after transfer is important to optimize pregnancy rates. It may be beneficial to keep the recipient mare in her original herd after transfer as opposed to moving her immediately to a different herd of mares. Anecdotal evidence suggests that social stress may adversely affect pregnancy rates in recipient mares.

Progesterone supplementation

It is probable that most embryo transfer recipient mares do not need supplemental progesterone. However, administration of exogenous progesterone or progestins to recipient mares following embryo transfer is routinely performed at some embryo transfer facilities and used sparingly or not at all at other facilities. The decision whether or not to supplement with progesterone is based on clinical experience,

value of the embryo and the perceived risk that luteal insufficiency may adversely affect embryonic survival in a given mare. Supplementation may include altrenogest (0.044 mg/kg; orally, once daily), short-acting progesterone (200 mg, IM, once daily), or long-acting progesterone (1,500 mg, intramuscularly, once every 7 days).

Progesterone supplementation may be discontinued at any time in early pregnancy provided that endogenous levels are measured and determined to be sufficient to maintain pregnancy (i.e. $\geq$ 4.0 ng/ml). Progesterone therapy may be discontinued between 45 to 70 days of pregnancy if an ultrasound examination confirms the presence of secondary corpora lutea or may be discontinued at 100 to 120 days of gestation without testing since the equine placenta produces sufficient progestins by day 90 to maintain the pregnancy.

An advantage of progesterone supplementation is that failure of maternal recognition of pregnancy (MRP) occasionally occurs in recipient mares, especially if the mare received a small, morula or early blastocyst stage embryo. Exogenous progesterone/progestin support can maintain a pregnancy in the absence of MRP.

Pregnancy examinations on recipient mares

Ultrasound pregnancy examinations are performed at very specific intervals based on embryo age (days 11, 12, 14, 16, 25 and 35). A majority of ET pregnancies can be detected by day 11 or 12. Small morula or early blastocyst embryos 150 to 250 µm in diameter may not become a visible embryonic vesicle in the recipient mare until day 14. The final check for early pregnancy status is on day 16 (embryo age). Early identification of pregnancy status allows for an early notification to an owner and facilitates subsequent breeding management decisions regarding the donor mare (i.e. rebreeding to the same stallion, switching to a new stallion or being finished for the season). Equine embryos grow at an average rate of 3 to 5 mm per day from day 11 to day 16 of pregnancy (Table 3). Failure to grow, reduced growth rate, or failure to advance in developmental stage may be associated with eventual embryo loss.

Empty trophoblastic vesicles

Empty trophoblastic vesicles (ETV) are occasionally noted after transfer of an equine embryo. An empty trophoblastic vesicle is defined as an embryonic (trophoblastic) vesicle without an embryo proper. An embryo destined to form an ETV often exhibits normal early growth (i.e. day 11 to 16). Ultrasonographically, an ETV is recognized as a static oval or irregular embryonic structure without an embryo proper after day 25 of gestation. Empty trophoblastic vesicles do not form endometrial cups, even if the vesicle is still present in the uterus after day 35. Once an ETV has been confirmed, the recipient mare should be administered prostaglandin to cause luteolysis and allow for a return to estrus. In a retrospective study of 820 embryo transfers at Colorado State University, 20 ETVs were detected on day 25 (embryo age), representing 2.4 % of all transfers and 3.3 % of day 16 pregnancies.

Pregnancy rate after transfer

Initial pregnancy rate

Pregnancy rate after transfer, pregnancy loss rate, and live foal rate are key statistics used to evaluate success of an embryo transfer program. Pregnancy data are difficult to compare among different publications, embryo transfer centers and years because of differing conditions and management systems. Table 4 is presented as a general guideline for initial pregnancy rates (i.e. day 16) after transfer of equine embryos in clinical practice.

Live foal rate

The ultimate outcome of a breeding program is birth of a live healthy foal. A retrospective study showed that there was no significant difference in live foal rate for embryo transfer recipient mares

pregnant at day 16 (144 of 171; 84.2 %) as compared to mares pregnant at day 16 carrying their own foal (116 of 134; 86.6 %).

Suggested reading

Hartman DL: Embryo transfer. In: McKinnon AO, Squires EL, Vaala WE, et al, editors. Equine reproduction. 2nd ed. Ames(IA): Wiley-Blackwell; 2011. p. 2871-2879.
McCue PM, Squires EL: Equine embryo transfer. Jackson(WY): Teton New Media; 2015. p. 1-172.
Riera FL: Equine embryo transfer. In: Samper JC, editor. Equine breeding management and artificial insemination. 2nd ed. St. Louis: Saunders Elsevier; 2009. p. 185-200.

Table 1. Embryo size on day 7 or day 8 after ovulation for mares bred with cooled or frozen semen.

Semen Type	Day 7 (µm)	Day 8 (µm)
Cooled	401.9 ± 19.6[a]	716.9 ± 104.9[c]
Frozen	258.2 ± 33.3[b]	383.5 ± 54.9[d]

[a,b]$p<0.05$; [c,d]$p=0.0553$

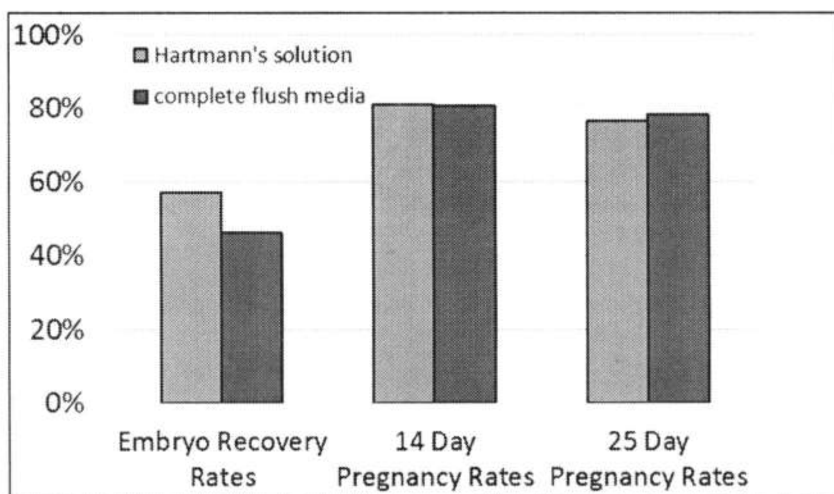

Figure. Embryo recovery and pregnancy rates following uterine lavage with complete flush medium or Hartmann's solution. There were no significant differences in either embryo recovery or pregnancy rates ($p > 0.05$).

Table 2. Embryo diameter (µm) relative to day of the collection procedure.

Collection Day	# Embryos	Mean ± S.D. (µm)	Range (µm)
6.5	20	191.8 ± 13.2	150 to 325
7	183	354.0 ± 13.9	150 to 900
8	35	623.9 ± 72.9	150 to 2,500

Table 3. Diameter of the embryonic vesicle in pregnant recipient mares on days 11 to 16 (embryo age).

Day of Pregnancy	Embryonic Vesicle Diameter (mm)
11	5.5 ± 0.1
12	8.5 ± 0.1
14	14.9 ± 0.2
16	23.5 ± 0.2

Table 4. Guideline for evaluation of success of an equine embryo transfer program.

Pregnancy Rate	**Evaluation**	**Comments**
≥ 90 %	Outstanding	Difficult to consistently achieve with large numbers of transfers
80 – 90 %	Excellent	Achievable with significant effort
75-80 %	Very Good	A solid goal
70-75 %	Good	Work on details
60-70 %	Fair	Need to improve
< 60 %	Marginal	Need significant improvement

How to add oocyte collection to your equine reproductive practice

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Introduction

There is an increased demand for intracytoplasmic sperm injection (ICSI) derived embryos in recent years in the equine industry. The increased popularity of this procedure is likely due to several factors including: increased familiarity of equine breeders with the procedure, an increase in personnel able to perform oocyte collection, and an improvement in the technique leading to increased success with the procedure. The following paper will outline the rationale for introducing oocyte aspiration for ICSI in clinical practice, and provide one facility's equipment, methods, results and clinical impressions regarding development and implementation of this procedure (transvaginal aspiration of oocytes from immature follicles) in a private practice setting.

Background

In vitro fertilization (IVF) in the horse is most commonly performed using ICSI. Traditional methods of gamete co-incubation for fertilization, as performed in bovine or human assisted reproduction is not reliably successful in the horse, with this difference due to low rate of sperm penetration through the equine zona pellucida.[1] Intracytoplasmic sperm injection was developed in the human field of reproduction to address male subfertility by allowing injection of sperm into the oocyte, with the first child born from ICSI in 1992.[2] The first report for commercial equine production of ICSI derived embryos was presented in 2007, this study reported a 58% oocyte recovery rate on transvaginal ultrasound-guided aspiration (TVA) of immature follicles, an average of 10 immature oocytes per aspiration session, a 12% blastocyst development rate per injected oocyte, and a 55% pregnant following transfer into recipients.[3] Several studies have followed which have reported an improvement in oocyte recovery from immature and dominant stimulated follicles (aspirated after administration of an ovulation inducing agent),blastocyst development rates and pregnancy rates following transfer.[4] The increased success of ICSI from TVA oocytes has prompted several practices to become proficient in the procedure of oocyte collection, with shipped oocytes making up more than half of the oocytes used for ICSI in some centers.

In general the procedure for the creation of ICSI derived embryos includes the following steps:1) recovery of oocytes from immature or dominant stimulated follicles,[4] 2) maturation (in-vitro for immature, in-vivo for mature) of the oocyte to a point when metaphase II (M II) of meiosis (intact oolema and visible polar body) has occurred and 3) fertilization (ICSI) performed by injection of an immobilized spermatozoan into the cytoplasm of the oocyte 4) culture of embryo for approximatelyseven days until the blastocyst stage is reached, and finally 5) transfer of the embryo in the blastocyst stage into a recipient mare. Several complete reviews of this process have been previously described, and the reader is referred there, as well as a discussion on the pros and cons of collecting immature versus mature oocytes for ICSI.[4-11]

The results of several commercial ICSI centers have been made available and have demonstrated reasonable efficiency of the procedure. Communication of reasonable expectations are based on these studies, and the client should be informed on the current expectations of success for each stage of the procedure to prevent unnecessary disappointment or expectations. In general research has shown that for oocyte aspiration in clinical practice a 50-70% recovery rate of each immature follicle aspirated should be expected, and an 80% recovery rate for dominant stimulated follicles (DSF).[4] For immature oocytes collected from normal mares, in-vitro maturation rate to the MII stage has been reported to be approximately 65%.[4,10] Following ICSI, the percentage of embryos that proceed to blastocyst range has been reported from 12-23%, and is highly dependent on the laboratory used.[3,4,10] Once the embryo has reached the blastocyst stage and has been transferred into a recipient, there is a reported increase in pregnancy loss in ICSI derived embryos with an expected 50-65% live foal rate. When explaining to the client what to expect, we usually inform them that that it will likely take two-three aspirations sessions,

recovering 8-12 oocytes to result in a live offspring. Obviously mare age, health, semen quality, shipping conditions and laboratory processing, recipient mare health and quality can all deleteriously effect these numbers.

The clients' mares that benefit from the option of obtaining of oocyte aspiration/ICSI derived embryos compared to traditional embryo transfer include the ability to bypass the uterus, oviducts, and abnormalities in ovulation. Mares with chronic uterine infections, suspected oviductal pathology, or mares that repeatedly have hemorrhagic anovulatory follicles and have had reproductive success prior to these pathologies make good candidates. In addition, for clients with limited access to semen (deceased stallion, subfertile stallion, limited semen available), ICSI derived embryos may be a reasonable alternative, as only one sperm is required for fertilization. In the case of untimely death of a mare, or impending euthanasia, oocytes can be harvested from the ovaries and used for ICSI as well.

The costs associated with the procedure are generally considered more expensive than traditional embryo transfer and for most practitioners the cost of oocyte aspiration will be separate from the cost of ICSI and recipient mare fees. In general we advise clients that the foal should be worth approximately $10,000.00 to justify the cost of the procedure. Comparison of several oocyte collection and ICSI centers have given the following range of costs:

1) Transvaginal ultrasound guided aspiration of immature oocytes (1 session: $500-$1,000)
2) Packaging and shipment overnight of immature oocytes (US domestic $300-$600)
3) Maturation, ICSI and embryo culture ($2,500-$4,000)
4) Transfer into recipient ($300-$700),
5) Cost of pregnant recipient mare ($1,200-$3,600)

Deciding if oocyte aspiration is right for your practice

The decision to proceed with the addition of oocyte aspiration in your practice will obviously vary with the individual practice. Addition of oocyte aspiration services allows a practice to provide clients the option of maintaining mares at home/nearby, and still having performing ICSI for reproductive management. The expense of the equipment required and the time needed to master TVA and achieve acceptable oocyte recovery rates, as well as access to mares to practice the procedure on, should not be overlooked. It is also vital to the success of setting up an oocyte aspiration service to establish a working relationship with a reputable ICSI center, one that is consistently achieving expected maturation, and blastocyst rates and is willing to assist in training or troubleshooting when needed. Many of the materials noted below were suggested in a previous review of how to start TVA in practice in 2013, but have been repeated here with some additional notes.[12] In addition, what follows is a very simplistic explanation of the materials and protocols involved in transvaginal ultrasound-guided aspiration of oocytes from immature follicles, much discussion and prior research has been conducted and is debated as to the optimal conditions, methods and materials to use, and the reader is referred to the resources listed as the end of the review for a more in-depth discussion.

Materials needed for TVA of immature oocytes

1) Ultrasound machine and transducer with the ability to achieve a reasonable image for transvaginal ultrasound guided aspiration. Often machines with poor resolution make it difficult to determine if follicles have been successfully entered with the needle, and air and artifact on some machines will make an already difficult procedure, fruitless and frustrating. In our practice we use an Sonosite M turbo with a micro-convex transducer (5-8 Mhz frequency range, 10 cm depth).[a] Others have successfully used a linear rectal transducer with fitted adapter for needle guide, that can be found supplied by several companies. The cost of an ultrasound machine ($6,500-$100,000),and microconvex transducer ($2,000-$12,000) ranges greatly and depends most on the quality of image you are willing to work with.

2) Transducer/needle guide. There are many variations of cases/guards that provide a rigid case to enclose the transducer and needle, maintaining a static relative position and the ability to manipulate the case easily to facilitate ovarian/transducer/needle positioning. It is important that the case/guard can be opened and the components properly cleaned between each aspiration, with gluteraldhyde being the disinfectant of choice.[b] These cases are often supplied by ultrasound companies, companies that supply TVA equipment, and some practitioners have even molded their own from plastic with success. The guide we use is a plastic pre-manufactured needle guide for a microconvex probe.[c] The cost of the needle guide ranges approximately from $200-$3,000.

3) Needle for aspiration. In our practice we use a 60cm, double-lumen,12 gauge stainless- steel oocyte aspiration needle.[d] One will also need to purchase a needle sharpener, that is recommended with the needle, to prevent dull or burred edges which make precise follicle puncture difficult. Cost is approximately $300-$500 per needle.

4) Aspiration source with foot controlled on-off and adjustable control of negative pressure.[e] The aspiration device should have a pressure relief valve and the ability to maintain 150-300 mmHg for follicular aspiration. The pressure should be monitored and should ideally not exceed 300mmHg. An aspiration source with foot pedal allows the aspirator and assistant to control when negative pressure is applied and prevents continuous aspiration when the needle is not in a follicle. Cost ($300-$2,000).

5) Plastic or glass collections bottles (250ml-500ml)[f], maintained at 37^0 C, with stopper.[g] Cost is $6-$60/container, depending on if you use disposable or glass and re-sterilize.

6) Tubing for connection of needle to collection vial and collection vial to vacuum.[h] Cost ranges from $40-$100, and disposable tubing is recommended.

7) Embryo transfer low-volume filter (75 micron pore diameter) is used to filter the collected follicular fluid following aspiration of all follicles. The filter is rinsed into a Petri dish and evaluated with a dissecting microscope for oocyte isolation.[i] All rinsing of flush dish and filter is performed using flush media and non-latex syringes. Cost of the filter dish can range from $15-$35, and some are resterilized and used again.

8) Dissecting microscope with 40x magnification ability.[j] A heated stage is recommended if one will be aspirating mature oocytes to ensure minimal fluctuations in temperature. Cost ranges from $2,000-$16,000, and is highly dependent on image quality desired.

9) Follicle flush medium is a standard embryo flush medium[k,l] with heparin 5 IU/mL added.[m] It is recommended to use a standard embryo flush medium with human medical grade heparin added. We use approximately 2-4 liters per aspiration session. Cost of flush fluid ($15-$35).

10) 20mL all plastic, non-latex syringes for flushing follicles and rinsing filter following oocyte recovery.

11) Oocyte holding medium: This medium will be used for oocyte holding and overnight shipment for immatuume oocytes. We use a standard embryo holding medium.[n] The cost of a single 6mL vial is approximately $3.

12) Oocyte shipping container:[o] Oocytes must be maintained (room temperature immature, approximately 37^0C for dominant stimulated/mature) at the same temperature during shipment to protect from temperature fluctuations. These incubators/shippers must be reliable and be able to

maintain a set temperature for a minimum of 24 hours. The cost of different incubators/shipping containers ranges from $2,000-$6,000 and though some use standard equine semen shipping containers, it is thought these are not adequate to hold temperature stable.

Method of immature oocyte collection

Mare preparation

Prior to the day of aspiration, the mare is evaluated by transrectal palpation and ultrasound to determine the number of follicles present. In general aspiration is attempted when the largest number of follicles (5-20mm) are present due to the higher oocyte recovery from follicles of this size, and that very large follicles can increase the difficulty of the procedure, and too few follicles will lower the number of oocytes potentially recovered. The mare is administered sedation (detomidine HCl 0.01-0.02mg/kg and butorphanol tartrate (0.01-0.02 mg/kg IV) and systemic antibiotics (ceftiofur crystalline free acid (Excede) 6.6mg/kg IM), immediately prior to entering stocks. Fecal material is evacuated from the rectum and transrectal ultrasound and palpation are performed, with all follicles >5mm measured and recorded. The mare is then administered *N*-butylscopalammonium bromide (0.9 mg/kg IV) to facilitate rectal relaxation, and the perineum, vulva and vestibule are aseptically prepared with Ivory™ soap.

Equipment set-up

The aspiration device/vacuum, collection vials, and the ultrasound with transducer are placed on a cart to the opposite side of the dominant palpation arm. The general set-up for transvaginal ultrasound guided aspiration of immature oocytes is depicted below, and letters denote sources of products listed in the footnotes above.

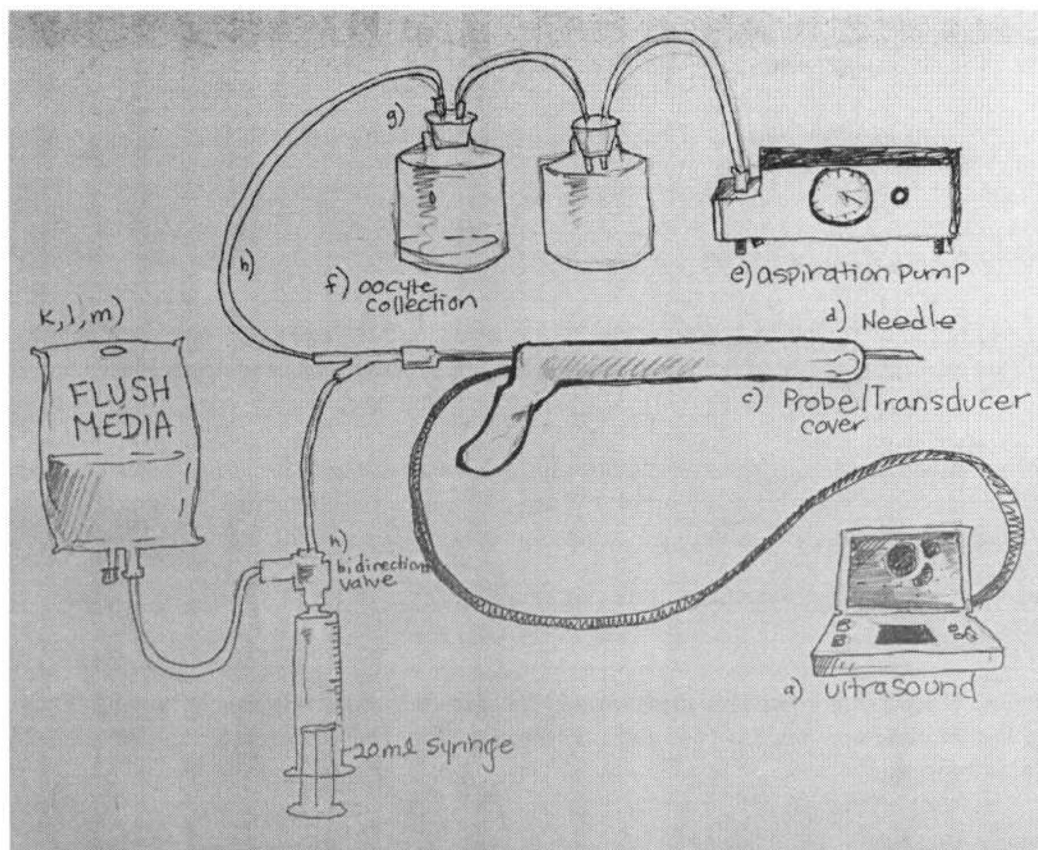

Figure. Drawing of general set-up, and equipment for trans-vaginal aspiration of immature oocytes.

Once transrectal ultrasound is completed with the linear transducer, the microconvex transducer is placed, in its case is changed into the ultrasound and the guard protected with a sterile sleeve. The person who will be performing the aspiration places a sterile sleeve and glove on and using copious sterile lubricant at the level of the transducer footplate, covers the probe with a sterile sleeve.

The needle is placed through the guard and flush medium with heparin is flushed through the needle and all tubing and containers flushed and primed so minimal air will enter the follicle upon aspiration. The needle is retracted into the guard to avoid trauma while positioning the case with transducer and needle in the anterior vagina.

Follicle aspiration

Sterile lubricant is applied to the hand and the guard with transducer is advanced through the vulva and vestibule to the anterior vagina. The footplate of the transducer is positioned to the 10 or 2 o'clock position of the anterior vaginal wall. Mild pressure is applied with the non-dominant hand to ensure contact with the transducer and vaginal wall and the image is monitored as the dominant hand is withdrawn and placed rectally for transrectal positioning of the ovary against the anterior vagina and transducer.

The follicle that will be aspirated is manipulated so that the needle, when extruded, will enter at the widest diameter of the follicle. Rotation of the probe and ovary together can facilitate positioning, and once ideal positioning occurs the hand holding the ovary can be used to brace the ovary against the probe to minimize movement. The needle is extruded into the follicle carefully by the same operator with the non-dominant hand and aspiration is initiated. Once the follicle has completely collapsed, fluid is pulsed by an assistant who is also controlling the foot pedal for aspiration pressure and the screen is monitored to ensure positioning. Complete aspiration and refilling of the follicle is performed ideally 5-10 times and then repeated another 5-10 times with the ovary manipulated and needle rotated to ensure scraping of the follicular wall at the time of follicular collapse. Once the follicle has been aspirated additional follicles in the same path of the needle are attempted, or the ovary is manipulated with needle still in place until another follicle aligns appropriately and the needle is then advanced into the next follicle. Negative pressure/aspiration is applied only when the needle is in the ovary or a follicle or when the lines are flushed to facilitate clot removal.

Filtration

Following aspiration all fluid is filtered through the embryo filtration cup and rinsed with additional flush fluid if hemorrhagic until fluid is clear. As much fluid as possible is removed and the contents of the dish are rinsed into a search dish, and oocytes are identified using the dissecting microscope.

Identification and packaging of immature oocytes

Oocytes are identified as characteristic, approximately 150 micron round oocytes with or without the cumulus cells at 40x magnification with a dissecting microscope. It is crucial to thoroughly tease out clots and debris as often oocytes become adhered and can be easily missed. Once identified, the oocytes are aspirated into a a glass micropipette or 0.25mL frozen semen straw, and transferred to a dish containing embryo holding medium (1-3mL). Ideally the holding medium is temperature matched to the flush fluid (by the end of the search this is room temperature), and all oocytes are placed in the dish until one is confident no more can be found in the initial search dish. The oocytes are transferred to a 5mL plastic Eppendorf tube containing fresh embryo holding medium, filled to within a few mm of the top, sealed in parafilm and packaged into the temperature controlled shipment container which is maintained and set at ambient temperature. The entire procedure from start to finish takes an average of 1-3 hours. Shipment is made for overnight delivery.

Results

Three boarded theriogenologists participate in oocyte aspiration in our practice. Two visited an ICSI center for training for 2-5 days within the first month of practice to receive guidance on the procedure, and one had prior experience. To train, each practitioner performed 5-15 aspirations on recipient mares prior to performing client aspirations. The first year 2 mares (average age 23 years) were aspirated with a total of 5 sessions with an average of 2.4 oocytes recovered. Two ICSI centers were sent oocytes and neither had a live foal produced.

The second year 12 mares (average age 16.6 years) were presented for oocyte aspiration with a total of 28 sessions with an average of 3.8 oocytes collected/session. Oocytes were sent to three ICSI centers and the number of live foals produced could not be verified. The third year 31 mares (average age of 18.4 years) were presented for oocyte aspiration with a total of 80 aspiration sessions with an average of 7.1 oocytes recovered/session.

Only one mare had complications associated with the procedure, as the mare was startled and jumped from the stocks with the vaginal probe in place and was euthanized to complications associated with the probe rupturing her aorta. Approximately 2-5% of the aspiration sessions will result in mild rectal irritation (evidence of blood on the sleeve), but no complications have been associated with this. No abscesses or systemic illness have been caused to our knowledge.

Discussion

The greatest advantage to adding this service to our practice is the flexibility it gives clients who want to pursue ICSI but do not want to move their mares at a remote site for oocyte aspiration. The greatest difficulty of providing this service was becoming proficient with the aspiration procedure, which was aided by the availability of recipient mares in the off season. One considering the addition of this service should expect a training period to master immature follicle aspiration, and close communication with an experienced and reputable ICSI center to ensure optimal results. The addition of this slightly time consuming procedure should be weighed in light of current case load, and client need, as often the time required to perform an aspiration can be very disruptive during the busiest time of the breeding season. The income provided by this additional service has paid for the equipment cost and is now a profitable service for our practice. In addition we have felt that the service has been beneficial to our clientele and ourselves as practitioners

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Footnotes

[a]UJIFILM Sonosite, Inc. Bothell, WA
[b]CidexPlus, Johnson and Johnson, Irvine, CA
[c]Needle guide for micro-convex transducer, Minitube of America, Inc, Verona, WI
[d]Aspiration needle: 60-cm, double-lumen, 12-gauge oocyte aspiration needle, Minitube of America, Inc, Verona, WI
[e]Equine Follicular Aspiration pump, Minitube of America, Inc, Verona, WI
[f]Oocyte 500mL collection bottles (19009/4107), Minitube of America, Inc, Verona, WI
[g]Non-latex stopper for collection bottle (19009/4102), Minitube of America, Inc, Verona, WI
[h]Tubing for Equine Oocyte Aspiration Set (19009/4101), and Equine Follicular Aspiration and Injection Device Tubing Set (19884/0614), Minitube of America, Inc, Verona, WI
Controlled Flushing Set CFS36, vented spike, 90cm tubing, and bi-directional valve, Mila International, Florence, KY
[i]Embryo Low Volume Filter (04137),ICPbio Reproduction, Spring Valley, WI
[j]Dissecting microscope, Olympus America Inc, Central Valley, PA
[k]EMCARE™ complete Ultra Flushing Solution, 2L , ICPbio, Reproduction , Spring Valley, WI
[l]Vigro Complete Flush, Bioniche Animal Health, Belleville, ON
[m]Heparin sodium injection, Sagent Pharmaceuticals, Schaumburg,IL
[n]EMCARE™ Embryo holding solution, 6mL vial , ICPbio, Reproduction , Spring Valley, WI
[o]Micro Q oocyte shipping incubator, Micro Q Technologies, Scottsdale, AZ

Equine ICSI, a private practice perspective: expectations, discussion, and thoughts

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Abstract

Embryo production by ICSI, especially through the use of transported oocytes, is becoming a more common clinical procedure in equine practice. Veterinarians and in turn their clients should become familiar with not only the logistics of the procedures but also factors that can influence the outcome in either a positive or negative manner.

Keywords: Embryo, ICSI, equine, OPU, TVA, oocyte

Introduction

The veterinary practitioner collecting and shipping equine oocytes for ICSI faces many challenges. The technique of oocyte pickup (OPU) by transvaginal follicular aspiration (TVA) presents mechanical and technical demands, but these are no more important than the challenges presented by the client and the practice situation itself. Embryo production by OPU and ICSI can yield remarkable results, but not all cases are equally productive. Our practice produced 450 commercial blastocysts via ICSI in 2016 with an average of .7 blastocysts per TVA, with a range of 0-7 blastocysts per TVA. Unfortunately, blastocyst production is not always distributed equally among clients and mares. Client expectations may or may not be realistic; unrealistic expectations can be difficult to satisfy leading to frustrations for clients and veterinarians alike. Horse breeders are familiar with the process of artificial insemination where the major obstacle is providing sufficient sperm to populate the oviduct in a timely manner so that an ovulated oocyte will be fertilized. When both ovulation and fertilization are performed with a mechanical nature via ICSI, the uninformed client will not see further barriers to successful production of an embryo, a pregnancy, a foal, and an eventual world champion. Understanding some of the processes involved can lead to more realistic expectations and an appreciation of success.

Attrition in the process

Immediately following recovery of oocytes the numbers of potential embryos visualized by the client starts dropping. Aspiration of small antral follicles in the mare draws oocytes from a diverse pool, especially in the first aspiration of the season for a mare. The oocytes can range from not being developed enough to enter meiosis when stimulated, to atretic and degenerated oocytes that no longer retain meiotic competence. This variety of developmental stages results in only a portion of the recovered oocytes responding to in vitro maturation (IVM), regardless of the efficacy of the IVM program. Some aspirations will yield a very high percentage of oocytes that respond to maturation, up to 100%, while others can have extremely low percentages of oocytes capable of meiosis. We see an average of 60-70% of the oocytes progressing to metaphase II, but each mare and each aspiration is unique, and while a client may assume all oocytes of an individual aspirate will mature, that may not be the case.

Removal of the cumulus cells surrounding the oocyte, denuding, is performed following IVM to allow visualization of the oocyte for evaluation and for sperm injection. This takes place in a solution of hyaluronidase that breaks down some of the bonds between cumulus cells themselves as well as between the cumulus cells and the zona pellucida. Each oocyte is repetitively pipetted through pipettes of decreasing diameter to remove the cumulus cells. Occasionally this process will result in breech of the zona pellucida, possibly due to zona pellucida damage during aspiration, resulting in lysis of the oocyte. This loss of oocytes in our laboratory is usually around 2-4% but can be significantly higher in certain instances.

Not all oocytes will survive the ICSI process itself. The equine oolemma is a remarkably elastic structure and is one factor that makes equine ICSI challenging. Its extreme elasticity resists penetration with a micropipette, and even when assisted with a piezo drill, penetration can result in tearing or damage sufficient to cause lysis to the oocyte. The oocyte will appear relatively normal immediately following

ICSI, but will start to degenerate in a short period of time. Oocyte lysis following ICSI is usually in the 2-4% range for our laboratory.

Oocyte activation is the next big hurdle in the general attrition of numbers. Metaphase II oocytes remain in meiotic arrest until activated by a cytosolic sperm factor, phospholipase C zeta (PLC zeta).[1] Release of sperm factor in the oocyte results in multiphasic calcium release that is necessary for the resumption of meiosis and eventual embryogenesis.[2] Stallion spermatozoa possess a relatively large amount of PLC zeta activity compared to other mammals, but individual stallions vary greatly in their capacity to activate oocytes in the in vitro environment.[3] While sperm cells from some individual stallions have high rates of oocyte activation others can have extremely low rates. Oocytes appear to vary in the ease of activation between mares as well, so the combination of a stallion and mare both with low activation rates can lead to extremely low activation and then cleavage of oocytes. We see an overall average of activation and then cleavage of sperm injected oocytes around 70%, but again, averages do not always hold true with individual cases.

Day 5 of embryo culture following ICSI offers the next dramatic reduction in numbers. This is the period when compaction occurs, creating the compact morula. Starting as a loose association the cells orient themselves into a compact mass with tight junctions. Cells that are not included often will lyse or be segregated out to become extruded blastomeres. Nearly all embryos that form compact morula in this phase, day 5-8 will progress to blastocysts in about two days, depending on the percentage of the cells included in the compact morula and the overall vitality of the embryo. Those that do not form compact morula will not progress. Average rates of embryos compacting at this stage are around 30%, but individual cases can vary from 0-100%. Counting on averages for individual cases can sometimes provide frustration for the client and veterinarian.

Oocyte quality/competence

Oocyte quality is probably the largest single contributor to the variability of success of equine ICSI. Meiotic competence, the ability to resume meiosis when freed from the inhibitory effect of the follicle is an important factor. Morphologic abnormalities such as vacuoles, misshapen oocytes, fragmentation, and evidence of aging such as thin zonas and large perivitteline space are negative indicators of quality. While one oocyte may appear like another, their innate potential can be quite different. The true measure of oocyte quality is developmental competence, the ability of an oocyte to not only complete meiosis but to respond to fertilization by activation, division, and development into a normal viable embryo. Depending on the individual case, the rate of developmental competence may approach that of meiotic competence or be far lower. Nuclear maturation can proceed more quickly than cytoplasmic maturation in the oocyte once placed in maturation conditions, and the inability of the cytoplasm to support the development of the resulting embryo appears to be an important factor. It also seems that there is further innate variability in the developmental competence of small follicle oocytes recovered from mare to mare.

Mare effects

Mare differences play an important role in blastocyst production, particularly antral follicle count, age, and oocyte quality. Antral follicle count will give some indication of the number of recoverable oocytes, and increased numbers of oocytes available for ICSI gives increased opportunity for successful embryo production. Antral follicle count will vary from season to season and when evaluating mares for aspiration we expect a higher oocyte recovery rate from follicles that are between 5 and 20 mm diameter with decreasing recovery from follicles larger than 20mm. One should also note that in some mares it is difficult to discern between subepithelial inclusion cysts commonly associated with the ovulation fossa and small follicles. The first aspiration session of the season has a tendency to yield higher numbers of atretic oocytes than subsequent aspirations.

Increasing mare age does have an impact on embryo production on the average, largely through decreasing antral follicle count and oocyte production. Age related oocyte quality effects seem to arise at different points for different individuals, but age 24 appears to be important in our clinical program. Each

year we have several 24-year-old mares that have good blastocyst and pregnancy production but after age 24 production falls, and early embryonic loss tends to increase.

Oocyte quality and developmental competence are quite variable from mare to mare. One factor that has been little discussed but appears to be important is the ease with which oocyte activation takes place.

Stallion factors

We find that some stallions' sperm has a high rate of oocyte activation, cleavage, and blastocyst formation compared to the average and other stallions have very low rates. While results from some of the low yielding stallions can be improved with sperm preparation and artificial activation, others can prove quite difficult. The proficiency of production by the sperm of a specific stallion can only be determined by use, either on clinical cases or via trials, something for a practitioner and client to take into account before starting on a project.

While only an individual sperm cell is injected into each oocyte, the overall quality of the sperm sample can have an impact on the outcome. Some stallion managers have reserved frozen semen of non-commercial quality for use on ICSI, saving the better freezes for commercial frozen semen AI. This can be counter-productive, as higher quality semen may give better results.[4] Since so little semen is used for ICSI, utilization of commercial quality semen should be suggested.

Refreezing previously frozen semen in ICSI-dose straws (usually around 1 million cells per straw) has become rather common and works well for some stallions. This can be a very useful procedure to increase the potential foals from some stallions with limited semen availability. Unfortunately it does not work as well for others; sometimes the sample does not retain good post-thaw motility following the refreeze and in some instances oocyte activation rates are decreased from that seen with the single-frozen sample. It is becoming more common for breeders to purchase refrozen ICSI-dose semen by the straw (especially for warmblood breeders) and practitioners should advise some caution. It would be prudent when possible to have some indication that semen from that refreeze batch has been used successfully.

Seasonal factors

Our practice has found the period of fall transition to be quite productive for some mares. These mares tend to have an increase in the number of small follicles available for aspiration and it appears that oocytes collected in this period can have an overall higher degree of developmental competence in some mares. Spring transition can also be useful, but the multiple follicles present do not remain small enough (5-20mm) to be high yielding for very long. As mares progress into the non-cyclic period of winter anestrus, useful oocytes can still be collected from most mares, but there appears to be a decrease in developmental competence of the oocytes collected, varying widely between individual mares.

In vitro embryos

Embryos produced by ICSI and in vitro culture are generally transferred to recipient mares as early blastocysts, roughly equivalent to a day 6 in vivo embryo, although this may occur between 6 and 10 days following ICSI. The embryos that form blastocysts earlier rather than later are more likely to establish viable pregnancies. Initial pregnancy rates in our practice are 5-10% lower than for in vivo produced embryos, and there is a higher rate of early embryonic death. Most of the pregnancy loss is before heartbeat detection, especially in slow-growing embryos, but there is a small group that will lose pregnancies between 30 and 45 days of gestation we do not generally see with in vivo-produced embryos.

In vitro produced blastocysts do not form an embryonic capsule until after they are transferred into the uterus. Since blastocysts that are left in culture too long will attempt to hatch by extruding trophoblast cells through the hole in the zona left from the initial ICSI, and since equine embryos not protected by a capsule or zona pellucida do not survive well in the uterus, there is a relatively small window of time in which the in vitro produced embryos are suitable for embryo transfer. The early blastocyst usually develops two days after forming the compact morula. This will often give some advance notice of an upcoming blastocyst and transfer for the practitioner, but sometimes the visually

dark nature of in vitro embryos combined with obstruction of visibility by extruded blastomeres or other cellular debris makes identification of the compact morula difficult. This means that practitioners receiving ICSI embryos for transfer might have less warning of the timing and number of embryos to be shipped than might be desirable. Once these early blastocysts are transferred, the first pregnancy examination is usually scheduled for fice days later, anticipating a 2.5-5 mm vesicle as would be normal for an 11 day pregnancy.

Conclusion

Many factors enter into the successful outcome of OPU of equine oocytes for ICSI, but even more factors can enter into a client's assessment of success and satisfaction for the process.

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An update on cooling and freezing extenders for stallion sperm

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Introduction

Methods for preserving stallion sperm, by cooling the sperm to 5°C or by cryopreservation, have been developing for >20 years.[1,2] Preserving sperm at 5°C, is usually accomplished by diluting sperm in a milk-based extender at room temperature and slowly cooling the sperm to 5°C using a passive cooling system.[2-4] The original milk-based extender was very simple and consisted of only dry skim milk, glucose, sodium bicarbonate and gentamicin sulphate.[5] Modifications off this main theme, including adding salts and proteins, have improved the survival of stallion sperm cooled to 5°C.

Most extenders originally developed to cryopreserve stallion sperm consisted of milk and/or egg yolk, with sugars, salts and glycerol.[6] Stallion sperm cryopreservation generally requires centrifuging the sperm, to concentrate the sperm and remove most of the seminal plasma; diluting the sperm in the cryo-diluent, packaging the sperm into straws and freezing the straws in liquid nitrogen vapor, either directly from room temperature or after cooling the sperm slowly to 5°C. Recent developments in both the freezing procedure and diluent composition have improved sperm cryo-survival rates. This review will update the practitioner on the recent changes made in diluents for cooling and freezing stallion sperm, as well as changes in techniques that improve the survival rates of sperm after cooling or after cryopreservation.

Cooled stallion sperm

Incubating sperm with high levels of seminal plasma, for extended lengths of time, is detrimental to stallion sperm. Therefore, sperm stored at 5°C, survive much better if the seminal plasma concentration is reduced to < 25% of the fluid volume. This can be achieved by diluting the sperm at least 1:3 with the cooling extender prior to cooling, or by centrifuging the sperm prior to mixing the sperm with the cooling extender.[3] Sperm from some stallions, do not cool very well, and removing the majority of seminal plasma (the final solution should contain 5% seminal plasma), from these samples, can sometimes benefit these sperm.[3] If the semen is centrifuged, care must be taken not to centrifuge the sperm with too much force or for too long, as this can damage the sperm. The use of a 'cushion' in the bottom of the centrifuge tube allows the sperm to be centrifuged with greater force and/or for longer times, which allows more sperm to be collected during the process, while limiting damage to the sperm.[3]

Modifications to the original skim-milk formula have produced a number of cooling extenders that exhibit improved sperm survival after cooling. A number of researchers have investigated adding different antioxidants to the media,[4] but for the most part these have not benefitted sperm significantly. However, some of the most promising additives to cooling extenders are additional milk casein proteins, including phosphocasienate.[2] Casein components are the main additional ingredients in the INRA96 and ARSBlue extenders, and sperm cooled to and held at 5°C in these extenders maintain higher sperm motility for 24-96 hours, than sperm diluted in the classic Kenney extender.[2] Cooling extenders with added casein products maintain higher sperm motility for sperm from most stallions, but seem to be especially effective for sperm that normally do not cool very well.

Cryopreserved stallion sperm

Cryopreserving stallion sperm typically involves centrifuging the sperm (to concentrate the cells and reduce the seminal plasma to ~5%), diluting the sperm into the cryo-diluent containing glycerol (2.5-5%) and packaging the sperm into straws. Depending upon the cryo-diluent used, sperm are often frozen in liquid nitrogen vapor directly from room temperature (lactose-EDTA extender; LEDTA) or first cooled to 5°C and then frozen (skim milk-egg yolk extenders; SMEY). Sperm are usually packaged into 0.5 mL straws, but straws up to 5 mL are sometimes used. The smaller, 0.5 mL straws are usually thawed in a 37°C water bath for 30 seconds prior to insemination, while the larger straws are normally thawed in a

50°C for 45 sec before being plunged into a 37°C water bath to stop the warming process, prior to insemination.

Again, many changes have been made in both the procedures used to cryopreserve stallion sperm as well as the diluents used to cryopreserve them. Both LEDTA and SMEY extenders contain various sugars in them, which act as non-permeating cryoprotectants. Studies have investigated whether sugars, other than lactose, can cryopreserve sperm better than lactose. However, these alternative sugars do not have major beneficial effects for cryopreserving stallion sperm.[7,8]

Although the most commonly used cryoprotectant, glycerol, permeates stallion sperm membranes; it does so rather slowly.[9] This means, when sperm are initially added to the freezing extender, that contains glycerol; and when frozen-thawed sperm are diluted from the freezing extender at insemination, water rushes out of the cell or into the cell, respectively; before the glycerol can slowly cross the membrane.[6] This causes the sperm to shrink or swell, respectively; and these cell volume changes can damage the cell (especially sperm swelling when the cryoprotectant is removed). These volume excursions can be lessened if cryoprotectants that are more permeable to the stallion sperm membranes are used, instead of glycerol. Experiments utilizing cryoprotectants that have lower molecular weight and that are more membrane permeable, than glycerol, such as methyl formamide and dimethyl formamide either alone or replacing part of the glycerol normally used in the cryo-diluent, result in higher post-thaw sperm survival than similar diluents containing only glycerol.[1,10]

Current dogma dictates that sperm frozen in LEDTA can be frozen in liquid nitrogen vapor directly from room temperature, while sperm frozen in SMEY should be first cooled to 5°C before being frozen. This suggests that sperm do not 'cold shock' in LEDTA, but are susceptivle to cold shock in the SMEY extender. Recent experiments have shown that sperm cryosurvival rates are higher in both LEDTA and SMEY if the sperm are cooled slowly to 5°C, to prevent cold shock, prior to freezing.[8] This simple procedure can easily be implemented into a cryopreservation protocol, to increase stallion sperm cryosurvival rates.

Finally, there is sometimes a desire to cryopreserve sperm collected from the epididymides of stallions that have recently died or that have been castrated. Currently, seminal plasma (collected from other stallions) is added to the epididymal sperm prior to cryopreserving the cells. In a recent study, we diluted epididymal sperm with either seminal plasma or a basic salt solution containing added sugars and protein, and found that sperm diluted in the salt solution exhibited higher post-thaw motility than sperm diluted in seminal plasma prior to cryopreservation (Graham; unpublished). This indicates that seminal plasma is not necessary for sperm cryopreservation.

Conclusions

Recent progress has been made in both the methodology and the extenders for cooling and cryopreserving stallion sperm. In particular a 'cushion' at the bottom of centrifuge tubes protects sperm from damage, while allowing greater centrifugal force and time to be used during centrifugation. This permits more sperm to be collected during the centrifugation process. New cooling extenders, which contain additional casein products, help stallion sperm survive the cooling process and storage at 5°C, better than extenders that do not contain added casein. Alternative cryoprotectants, which are of lower molecular weight than glycerol, create less osmotic damage to sperm, during their removal and result in higher cryosurvival rates, than glycerol alone. Epididymal sperm can be cryopreserved using the same methods and cryo-diluents as ejaculated sperm, but do not need to have seminal plasma added to them after collection. Diluting the epididymal sperm first in a basic salt solution containing sugar and protein is sufficiently effective to activate sperm motility and permit sperm concentration to be determined prior to diluting the sperm in a cryo-diluent. Finally, sperm should be cooled to 5°C before being frozen.

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Collection, management and distribution of frozen equine semen

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Introduction

Over the last 30 years the use of frozen semen in the equine industry has increased significantly and has become a relatively common method for producing horses of numerous breeds. The increased use of frozen semen can be attributed to improvements in techniques for semen cryopreservation, availability of reliable ovulation induction drugs, increased education of practitioners on the use of frozen semen, simplified mare management techniques and major registry acceptance of foals conceived by frozen semen. Additionally, adherence to quality standards for commercial distribution has led to more positive outcomes for mare owners and veterinarians which in turn has resulted in a greater acceptance of the technique as a viable method of breeding.

Practices that wish to provide a high quality, comprehensive frozen semen service for their clients should be experienced with methods to optimize the quality of collected semen, maximize retention of semen quality through the cryopreservation process and during storage and distribution. This review will reflect on our experience and recommendations in this field.

Optimizing quality of collected semen

While it may seem to be a very elementary point, the first and most critical step in obtaining good quality frozen- thawed semen is to start with a good quality fresh semen sample. However, in my experience even experienced clinicians often have poor results freezing semen due to starting with samples that are adversely affected by improper stallion management, poor semen collection or semen handling techniques and poor hygiene protocols. Since the spermatozoa will be subjected to significant stresses during cryopreservation and thawing, any sub-lethal latent damage to the cells caused by improper handling will likely result in an inability of those cells to withstand these stresses.

Stallions that are sexually rested tend to store aging spermatozoa in their extragonadal sperm reserves and these aged sperm have reduced quality. Therefore, when collecting ejaculates for cryopreservation one should first deplete stored sperm reserves through a series of "clean-out" collections followed by one or two days' sexual rest before collecting for cryopreservation. The number of ejaculates required to fully deplete sperm reserves varies among stallions (generally two to five is sufficient) and we recommend three successive daily collections prior to attempting to freeze. The semen quality and sperm output should be monitored and typically total sperm number per ejaculate will decrease and semen quality (both motility and morphology) will increase and become stabile as the stallion's aged sperm are depleted. Once this is done we find every other day or three times per week schedule to be best for ongoing collections of semen for cryopreservation. Although this is a general rule of thumb some stallions will tend to rapidly and significantly accumulate sperm in the reproductive tract and may require many more and very frequent depletion collections to provide the best quality spermatozoa. It is also my experience that some stallions will produce the highest post-thaw quality when collected on a daily schedule despite no observable difference in fresh semen quality when compared to less frequent collections.

Because seminal plasma makes a poor culture medium for sperm, the ideal type of ejaculate for semen preservation, either cooled or frozen, is one which has a low volume and high sperm concentration. While total sperm output is relatively constant for stallions on a regular collection schedule, seminal volume and therefore sperm concentration in the semen is greatly affected by the amount of fluid contributions from the accessory sex glands. The amount of accessory sex gland fluid in the ejaculate is influenced by the degree of sexual stimulation prior to ejaculation. Ideally, the pre-collection stimulation should be just enough to cause the stallion to ejaculate on a single mount but not so much as to cause an excessive amount of accessory sex gland fluid. Efforts should be made to deflect the stallion's penis away from the artificial vagina to allow for the voiding of pre-ejaculatory secretions which for some stallions can contribute significant sperm-free fluid to the ejaculate volume. In a large retrospective study

of pre-freeze and post-thaw semen quality data from semen frozen by SBS laboratories Kalmar et al reported significant correlations between the number of mounts required for ejaculation and seminal characteristics. As the number of mounts required for ejaculation increased, seminal gel-free volume increased and both sperm concentration and initial total and progressive motility decreased. More importantly, as initial sperm concentration decreased, post-thaw total and progressive motility decreased ($p<0.001$). Multiple mounts also lead to greater risk of sample contamination with pre-ejaculatory fluids and lubricant as well as debris and bacteria from the external genitalia and abdomen of the stallion. Significant loads of contaminating bacteria will adversely affect initial and post-thaw semen quality and so proper hygiene during collection and when handling semen is critical. In a study on the prevalence and type of bacteria in extended chilled semen (Althouse), 66% of commercially prepared semen samples received by a group of clinics in the US were found to contain significant bacterial contaminants despite the antibiotics included in the commercial extenders. This illustrates the apparent lack of proper hygiene and sanitary protocols and antibiotic selection in the horse industry.

Maximizing post-thaw semen quality

Maximizing post-thaw semen quality from each individual stallion is critical to obtain consistently acceptable fertility with frozen semen. Damage from freezing and thawing can be attributed to destabilization of sperm membranes as cells move to and from storage temperatures (thermal stress) and osmotic stresses created during freezing and thawing. When external pure water in the extender freezes, the result is an increased concentration of solutes outside the cell creating an osmotic gradient between the inside and outside of the cell. Unfrozen water within the cell then moves across the cell membrane out of the cell to balance the osmotic pressure resulting in a decrease in cell volume and dehydration of the cell. This dehydration exposes the cell to high concentrations of solutes within the cell which can damage cell membranes. Additionally, as temperature drops, the lipids within the cell membranes undergo a phase transition from a more fluid liquid state to a more rigid gel state. Membrane proteins and phospholipids can reorganize in a way which leads to alterations in membrane permeability to water and ions, premature capacitation and eventual cell death.

Penetrating cryoprotective agents (CPA's) such as glycerol, DMSO, ethylene glycol and amides are added to semen freezing extenders to minimize the damaging effects of high solute concentrations and intracellular ice formation. However, addition and removal of these penetrating CPA's from the cells leads to large changes in cell volume as water moves into or out of the cells to balance the gradient in CPA across the cell membrane. The most common CPA in equine semen extenders is glycerol which has a permeability across the plasma membrane much lower than water, therefore glycerol moves into the cell after dilution in freezing extender at a much slower rate than water moves out leading to further dehydration of the cell during cooling/freezing. Conversely, when frozen sperm are thawed and placed in an environment free of glycerol (non-glycerol containing diluent for evaluation or mare uterine fluids after insemination), water in the environment moves into the cell much faster than glycerol diffuses out leading to rapid cell volume increases and disruption of cell membranes.

Lipid and protein sources such as egg yolk and milk are also common ingredients of semen extenders for their membrane stabilizing and antioxidant properties. Another major component of extenders are sugars such as glucose, lactose, raffinose, etc. which provide an energy source as well as act as non-penetrating CPA's.

Species differ in the susceptibility of their sperm to damage due to cold shock and cryopreservation. These differences are thought to be related to the biochemical structure of the plasma membranes, specifically the cholesterol:phospholipid ratios, fatty acid content and membrane fluidity. It is believed that these differences are likely responsible for the variations in osmotic stress tolerance seen between species whose sperm survive cryopreservation well versus those that do not.

In addition to this species-specific variability, a well-documented inherent variation exists between individual males of many species in the ability of their sperm to withstand the stresses associated with freezing and thawing (cryotolerance). This male to male variation is especially evident in stallions. In dairy cattle, bulls have been selected by the AI industry for more than 50 years based on the ability of

their sperm to withstand the stresses of standard cryopreservation protocols. This selection has led to an increasingly uniform and positive response to cryopreservation. Studies on membrane fluidity and osmotic stress tolerance have demonstrated that bull sperm have a much greater tolerance for exposure to hypertonic conditions than stallion sperm and that there was a three-fold greater variance in osmotic stress tolerance between individual stallions than between individual bulls. Studies with boar sperm and human sperm have also revealed significant male to male variation in plasma membrane composition and some correlations have been found between cholesterol to phospholipid ratios, membrane fluidity, fatty acid content and response to cryopreservation. Further evidence for the relationship between membrane composition and cryosurvival comes from experiments with four different strains of mouse sperm that vary significantly in their cholesterol:phospholipid ratio. The percentage of motile sperm after thawing was directly correlated with the cholesterol:phospholipid ratio. The researchers were also able to dramatically improve cryosurvival in the low cholesterol strain by increasing the cholesterol content of the sperm membranes with cholesterol loaded cyclodextrins.

To date there is no single universal cryopreservation protocol that is optimum for semen from all stallions and use of a single protocol (extender, cooling rate, etc.) However, it is erroneous to group stallions into "good" and "bad" freezers based on post-thaw evaluation of semen frozen using a single common protocol. Our belief is that semen from a large percentage of stallions in the population can be frozen successfully if an effort is made to customize cryopreservation protocols to identify optimum conditions for each individual stallion. This has led to the practice of performing split-ejaculate test freeze procedures utilizing different extenders and cooling rates when evaluating a new stallion presented for freezing. It is important that the different protocols are tested on split ejaculates rather than comparing the results from one technique one day and another technique the next.

Our goals are to:

1) Produce the highest quality frozen semen from every individual stallion, not just accepting what appears to be adequate based on results from a single standard protocol and
2) Identify conditions for genetically desirable individual stallions deemed to be "poor freezers" that allows them to be included in commercial frozen semen breeding programs.

Our approach is to employ multiple protocols that are designed to determine the optimum procedure for maximum retention of semen quality after thawing of frozen semen from each individual stallion. The various extenders we use employ different sources and amounts of lipids, proteins, sugars and various penetrating and non-penetrating cryoprotectants designed to control damaging cell volume excursions during freezing and thawing.

Recently, we conducted an extensive retrospective study of data collected from stallions presented to two SBS laboratories in the United States that underwent split-ejaculate test freeze procedures during the years 1997 to 2016. The data included 1578 test freeze procedures on 1210 individual stallions of a wide variety of breeds and ages. Collected ejaculates were split into two to four fractions and frozen in 0.5 ml straws per the standard cryopreservation protocols for each individual treatment using a controlled rate cell freezer. Pre-freeze and post-thaw motility was evaluated using computer assisted semen analysis (CASA). Test straws from each treatment were thawed, diluted to approximately 25 million/ml in an appropriate extender and incubated at 37°C for 30 minutes prior to CASA analysis. Progressive motility was defined as the percentage of sperm that exhibited an average path velocity (VAP) > 50 mic/second and a straightness ratio (STR) > 75%. An ejaculate was considered "acceptable" for commercial distribution if post-thaw motility was ≥ 30%.

Overall, 81% of ejaculates subjected to the test freeze procedure resulted in acceptable post-thaw motility of 30% or greater in one or more of the extender treatments tested. If only one of the most common protocols had been used for these ejaculates only 64% of the freezes would have resulted in acceptable post-thaw progressive motility ≥ 30%. Therefore, an additional 17% of stallions were frozen successfully when the split-ejaculate test freeze method was used to select an optimum protocol.

Additionally, there was an average increase of 10 percentage points in the post-thaw progressive motility when the optimum protocol was selected versus the single standard protocol.

Post-thaw quality assessment and standards

When producing doses of frozen semen, the processing laboratory has an obligation to both the stallion owner and their mare owning clients to provide an accurate and objective representation of the quantity and quality of semen in the doses produced. While there is no officially recognized quality standard for equine frozen semen, the generally accepted industry standard is that an insemination dose of frozen semen should contain a minimum of 200 million progressively motile sperm with $\geq$ 30% progressive motility. We also recommend that the semen be free of known mare pathogens or a heavy load of contaminating bacteria. Since so much equine frozen semen is now being sold by the dose with little or no guarantees of fertility or quality it is important that mare owners can be confident they are purchasing doses that, at a minimum adhere to some quality standard as assessed by objective methods. This information should be available to mare owners and veterinarians so that owners may make informed purchasing decisions and veterinarians can make recommendations and management decisions to maximize the chances of success.

Sperm numbers per insemination dose must be determined accurately which requires a measurement of sperm concentration in the extended semen following centrifugation and resuspension with the freezing extender prior to packaging in straws. Because the semen is now extended in freezing extender, a standard photometer cannot be used and so a direct counting method such as hemacytometer (minimum of four chambers), Nucleocounter SP100 or CASA should be employed. Post-thaw sperm motility when determined subjectively is highly variable between laboratories and among technicians and is very subject to bias by the observer. Ideally, an objective method of determining post-thaw motility such as CASA is employed to minimize technician bias. The conditions under which the samples are evaluated is also an important consideration as variations in conditions will affect the measurement of motility. Variables that can affect measurement of post-thaw sperm motility (both subjective and objective) include; sperm concentration, diluent type, extender clarity, incubation time and temperature, stage temperature, quality of optics, chamber or slide type and technician experience. If an objective CASA system is used, the analysis parameters can be set by the user and so framing rate, number of frames analyzed and numerous other variables in the algorithms may vary between manufacturers and between two labs using the same system with different settings. Additionally, the velocity and straightness thresholds which determine if a motile cell is progressive or not may be defined by the user and differ between two laboratories. Therefore, when reporting motility information, the method of analysis and analysis parameters should be stated.

Semen storage

One of the main advantages of frozen semen is that when properly maintained at liquid nitrogen temperature (-197°C), spermatozoa can remain viable for an indefinite period, provided the spermatozoa respond favorably to the freezing process. Bovine semen stored frozen for more than 40 years has been reported to achieve pregnancies and personnel in our laboratory have used equine semen frozen for more than 30 years with good results. To maintain maximum fertilizing potential, spermatozoa must not be exposed to fluctuations in temperature and must be maintained below the critical temperature (approximately -130°C) at which thermally driven chemical reactions proceed. Exposure of cells to temperatures above -80°C can initiate some degree of thawing and then re-crystallization of ice, as well as reorganization of membrane proteins and lipids. This can lead to cell damage and impaired fertilizing potential. To maintain cells at these ultra-low temperatures, packaged frozen semen is typically immersed in liquid nitrogen. Specialized, insulated cryogenic containers have been developed that allow for such storage.

The most common type of cryogenic containers used for semen storage in the equine frozen semen industry are the smaller (20-50 liter), insulated, double walled, aluminum containers. The containers function by providing a chamber into which liquid nitrogen is loaded and the packaged semen

is immersed. Containers are manufactured by several companies and can be purchased in a variety of sizes to accommodate specific requirements. In liquid form nitrogen is extremely cold (-197°C/-320°F) and, when left in an open container, evaporates quickly into nitrogen gas. Cryogenic containers are designed to minimize the rate at which liquid nitrogen is converted to gas and allow for ultra-low refrigeration of the samples without electrical requirements. The inner chamber is constructed of aluminum which is then wrapped with an insulating foil. A second aluminum chamber surrounds the inner chamber and foil and the two chambers are welded together at the neck of the container and vacuum sealed. This construction allows temperatures within all parts of the container to remain lower than -180°C by circulating nitrogen gas within the chamber even when the level of liquid drops to a few centimeters. These tanks have round canisters with handles that hang down into the chamber from the neck of the container.

The following are recommended guidelines for the safe storage of frozen semen in liquid nitrogen containers.

1. Aluminum nitrogen containers should not be stored directly on concrete floors as this will erode the aluminum.
2. Place storage containers in a well-ventilated space, because escaping nitrogen gas will displace oxygen in a confined space and breathing air that is less than 18% oxygen could lead to a life-threatening situation.
3. Place storage containers in a room that allows for frequent visual inspection. A container that loses vacuum suddenly may exhibit a frosting around the neck of the container. If observed quickly enough it may be possible to move the semen to another container before all the nitrogen is lost.
4. Protect containers from impact as sudden impact could break the seal between the two chambers of the container and lead to a loss of vacuum and rapid evaporation of nitrogen within the container.
5. Check liquid nitrogen levels with a ruled stick at regular intervals and record the level. In our laboratory, nitrogen levels are checked weekly and nitrogen consumption recorded. A container that is starting to lose vacuum will be less efficient and increase the rate of nitrogen consumption. Detection prior to complete vacuum failure allows for the semen to be safely moved to another container.
6. Use caution when topping off containers with liquid nitrogen because the plastic straws at this temperature are extremely brittle and can easily crack causing damage that may not be revealed until the straw is thawed.
7. Prevent liquid nitrogen or objects cooled by liquid nitrogen from contacting bare skin as it can cause severe frostbite almost instantly.
8. Wear gloves and protective eyewear when handling liquid nitrogen.
9. Always dispose of liquid nitrogen outdoors by slowly pouring onto the ground and allowing the nitrogen to vaporize into the atmosphere.
10. Do not seal containers tightly as pressure build up from the expanding gas may lead to an explosion if not allowed to vent.
11. Always use a solid wooden, metal, or plastic dipstick to measure liquid levels. Never use a hollow rod or tube as the gasification and expansion of the rapidly cooling liquid inside the tube will force liquid to spurt from the top of the tube.

Accurate inventory management is critical for any storage facility or veterinary clinic holding frozen semen for clients. Small inventories can be easily tracked using a manual paper, index card or spreadsheet based system. For larger storage facilities that have a lot of inventory being added and removed for distribution, a computerized database system is useful. The key to any system is a strict policy of recording transactions that accounts for each individual straw of semen in storage. This requires that all straws entered into storage are counted accurately and that all data pertaining to those straws be entered as well. Straws should be labeled permanently with the stallion's name, registration number,

freezing facility code or name and date of freezing or coded lot number. It is highly recommended that pre-printed straws be used rather than hand labeling as the handwriting on straws is often illegible and can lead to confusion concerning the identity of the semen. All this information as well as the semen owner, sperm concentration in the straws, number of recommended straws per insemination dose, post-thaw quality and exact location (container, canister number and section within the canister) for each individual lot should be included with the inventory.

Frozen semen distribution

Frozen semen is typically transported in a nitrogen vapor-phase container (dry shipper). These cryogenic containers maintain near liquid nitrogen temperatures (typically -180 to – 195°C) for days or weeks without the use of hazardous liquid nitrogen. Vapor phase containers work by absorbing liquid nitrogen into a thick layer of hydrophobic absorbent material that surrounds the inner cavity of the container where the semen is stored. A long holding time is achieved through the superior insulation afforded by the double-walled aluminum shell that is filled with insulating foil and vacuum sealed. Vapor phase containers must be properly "charged" by filling with liquid nitrogen to the point of saturation of the absorbent material and then pouring off the excess liquid nitrogen. Such vapor-phase shippers can be transported with a "non-hazardous" classification throughout the world, which significantly reduces transport costs. A word of caution is offered, however, concerning the use of vapor shippers; since the package is being shipped with a non-hazardous classification, it is critical that all the liquid nitrogen is poured out of the container prior to transport. Transport of a hazardous material without the proper declarations and appropriate labeling can lead to significant fines as this is a violation of IATA (International Air Transport Association) regulations and may be in breach of certain state and federal laws. Vapor shippers can be used to store semen for longer than the typical recommended holding time if more liquid nitrogen is added. If a practitioner receives semen in a vapor shipper in anticipation of inseminating a mare and she does not develop a pre-ovulatory follicle as anticipated or she ovulates before the semen arrives, the vapor container may be used to store the semen for an extended time provided the vapor container is filled again with liquid nitrogen. If this occurs, the practitioner should contact the semen supplier as soon as possible to discuss the shipper's policy regarding tank returns.

Vapor shippers are fairly durable however it is highly recommended that they be placed inside a solid outer shipping carton that will protect the container against physical damage or loss of vacuum due to rough handling during shipping. A commonly used shipping carton has a round top and is base wide to help keep the shipper upright and prevent the package from being transported upside down or on its side. Even though it is a vapor shipper, if left on its side or upside down, the nitrogen gas will "pour" out of the container and significantly reduce holding time. Eventually, vapor shippers will lose vacuum resulting in a loss of holding time. Prior to the beginning of each breeding season it is highly recommended that all shippers be properly tested to determine if they are holding temperature per specifications. Vapor shippers and liquid storage containers that have lost all or partial vacuum can often be repaired by the manufacturer.

When packaging frozen semen for shipment, extreme care should be taken to avoid exposing straws to elevated temperatures. Use of an accurate and convenient inventory system allows the technician to quickly locate the correct straws to be loaded without having to search through canisters, trying to identify the appropriate straws. If large 5- or 4- ml straws are to be shipped they are simply loaded into the canister of a fully charged vapor shipper, taking care to always work within the neck of the storage container below the frost line as previously mentioned. Lower the frozen straws gently into the canister to prevent cracking and pack the empty space around the straws with cotton to keep them from moving inside the canister during shipment. For semen packaged in 0.5-ml straws there are two options. Straws can be loaded into small goblets and placed on canes or they can be loaded into larger plastic goblets and shipped in bulk. If straws are stored in bulk, then shipping on canes requires that the individual straws are loaded into the smaller goblets and clipped into the canes while working under liquid nitrogen. This can be accomplished with a small thick walled poly foam box filled with liquid nitrogen, under which the straws, goblets and canes are handled. While under liquid nitrogen the

technician can easily verify the identity of the semen by reading the printed straws. Printed straws held in nitrogen vapor often become frosted making them difficult to read. Straws and goblets should be handled with pre-cooled tweezers or hemostats. Likewise, when shipping in bulk, all transfers from the storage goblet to the shipping goblet should take place under liquid nitrogen. Lifting individual straws into room temperature air to transfer from liquid storage to vapor shipper should be avoided. Straws packaged in large goblets for bulk shipment should also have cotton packed around them to prevent cracking.

To facilitate proper use of the frozen semen and provide the veterinarian with information needed to manage insemination of the mare one should include specific instructions on thawing and handling the frozen semen as well as a transaction report or shipment form with the shipment. The transaction report should include the stallion's name, number of doses, number of straws per AI dose, collection date or lot number, number of total and progressively motile sperm expected upon thawing, semen EVA status, mare's name, breed and registration number if available and the mare owner's information.

Additional information that may be required or useful includes; instructions for return of the container, policy regarding unused doses and applicable breed registry insemination or distribution certificates.

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Clinical perspectives on the stallion

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Introduction

Stallions are presented to Select Breeders Service laboratories for a variety of reasons including; training for semen collection, breeding soundness examination, semen evaluation and cooled and frozen semen processing. This review will outline the process that we use when presented with a stallion for semen collection and processing of frozen semen and our approach to troubleshooting stallions that exhibit abnormal semen characteristics or poor results from standard preservation protocols.

Standard workup for new stallions

As a facility approved for semen collection and storage for the European Union and a variety of other countries that is inspected twice yearly by the USDA, we have implemented some basic health screening requirements for all stallions entering our facility. These include a negative EIA within 12 months and negative EVA and CEM within 30 days of entry. Stallions seropositive for arteritis virus must show proof of vaccination (and appropriate boosters) or have a negative virus isolation on the semen to confirm that they are not shedding virus in the semen.

Our approach for the first collection attempt is different for a stallion that has previous experience mounting a phantom than it is for a novice. Briefly, if it is a novice stallion, we will introduce him to the breeding shed environment without a stimulus mare, allow him to see and approach the tease rail and the phantom and get comfortable with the environment before bringing in an estrus stimulus mare. We then introduce him to the stimulus mare to elicit an erection and wash the penis with warm water and cotton and at this time examine the external genitalia for any abnormalities. Testicular palpation and measurements are performed after successful collection. Novice stallions are then brought to the phantom with the stimulus mare positioned alongside and attempts are made to encourage the stallion to mount the phantom. This process may take ten minutes or several days depending on the stallion but the key is patience on the part of the stallion handler and collector and consistent positive reinforcement during the training process.

For experienced stallions, we will generally start with a stimulus mare in the shed and take them straight to the phantom after washing, with or without a stimulus mare positioned near the phantom based on the stallion's behavior and libido.

Prior to commencing on a freezing program with a new stallion we will collect and evaluate the semen. If the stallion is sexually rested or if we have no real history, we plan on performing a minimum of three daily collections to deplete extragonadal sperm reserves and evaluate semen quality. If the semen quality is satisfactory and stabilized after three collections, we then give the stallion one day of sexual rest and perform a split-ejaculate "test-freeze" procedure. If sperm numbers are not stabilized or semen quality is poor (low progressive motility and/or poor morphology) we may continue to deplete the stallion's sperm reserves until they are stable. For standard evaluation, sperm concentration is measured using a Nucleocounter, sperm motility is evaluated using a computer assisted sperm analyzer (CASA) and morphology is assessed on stained samples of 200 total sperm using 1000X oil immersion phase contrast microscopy.

Basic test freeze procedure

The number of protocols evaluated in a typical split-ejaculate test freeze depends on the total number of sperm available. If sufficient sperm are available, we will evaluate four different protocols in the first test freeze. The protocols differ in the extender used and the cooling/freezing rate employed. If there are not sufficient sperm in the ejaculate to evaluate all four treatments we will use two or three, select the treatment that provided the best post-thaw motility and then compare that treatment to additional treatments in subsequent split-ejaculate test freezes. Following determination of concentration and motility, semen is diluted with a centrifugation extender and centrifuged on a cushion in 50 ml

conical bottom tubes. The supernatant is then removed as well as the cushion fluid and the sperm are re-suspended in the appropriate freezing extender. The concentration of sperm in the extended semen is again counted using a Nucleocounter and final adjustments are made to achieve the desired concentration before loading the semen into 0.5 ml straws. Straws are sealed, racked and frozen using a controlled rate cell freezer. For each treatment and all ejaculates, two straws are thawed and evaluated. The contents of the two straws are combined in a sterile tube and a volumetric loop is used to obtain a sample for bacteriology on a blood agar plate. An aliquot of the thawed semen is diluted to approximately 25-30 $x10^6$ per ml with pre-warmed extender (of the same type used for centrifugation of the raw semen) and the extended semen is incubated at 37°C for 30 minutes before being analyzed for motility using CASA. The technician performing the motility analysis is blinded as to the stallion and treatment and post-thaw evaluations are done in batches which include different stallions and treatments to minimize the potential for bias. Based on the results of the test freeze procedure(s) one of the protocols is selected and used for subsequent freezes on ejaculates typically collected three times per week until the desired number of acceptable doses is achieved.

Troubleshooting the "problem stallion"

The following sections describe our systematic approach to addressing abnormal findings in our basic semen evaluation or initial freezing attempts to design a customized program that achieves acceptable results for the stallion.

Observation: low sperm numbers

When low total sperm numbers are observed in the collected semen one should first determine if the sperm output is or isn't consistent with the expected sperm production based on examination of the testicles and calculation of testicular volume. If the low numbers are due to small or degenerated testicles, then there is very little to be done to increase sperm production. If sperm output is less than expected based on testicular volume and there are no abnormalities observed in the testicles upon ultrasound examination, then possibilities include incomplete ejaculation, retrograde ejaculation into the bladder or spermiostasis (blockage of sperm in the reproductive tract), typically in the ampulla. If the sample contains no sperm but the stallion appears to have ejaculated, then it can be assayed for the level of alkaline phosphatase, a testicular component of the ejaculate. If alkaline phosphatase is low, then a complete blockage is suspected. If high, then the semen does contain the testicular contribution and severe testicular dysfunction is suspected. Palpation or ultrasonic examination of the ampullae per rectum can often detect enlarged ampullae with distended lumen. Treatment for blocked ampullae is repeated collections sometimes aided by aggressive massage of the ampullae per rectum and administration of oxytocin (10-20 IU) or cloprostenol (25-125ug) given 5-10 minutes before semen collection.

Observation: excessive gel fraction in ejaculate

The gel fraction is typically the last component of the ejaculate and is contributed by the seminal vesicles. Some stallions produced little or no gel fraction while others produce a consistent 5 to 20 ml of viscous gel that is easily filtered out using an in-line nylon gel filter. Occasionally, a stallion is presented that produces copious quantities of gel and sometimes this gel is not easily filtered and can mix with the gel-free portion of the ejaculate. Semen which contains thin gel that is not filtered out is very difficult to accurately pipette and analyze sperm quality and in some cases may adversely affect sperm quality. Like other accessory sex gland fluids, excessive stimulation tends to increase the amount of gel produced. In these cases, management should minimize stimulation of the stallion by housing him where there is little other horse activity and schedule collecting the stallion early in the day before any other stallions are collected. When this fails to alleviate the issue the ejaculate can be fractionated and only the gel-free jets collected which prevents the gel fraction from mixing with the semen and usually results in improved semen quality.

In some rare cases the contaminating gel is very toxic to the sperm causing a rapid loss of sperm motility. Below is a description of one such case of extremely toxic and difficult to filter gel which prevented this stallion from being used in a cooled or frozen semen AI program.

Our approach was to use an open-ended artificial vagina for collection which allowed for the stallion's glans penis to protrude through the back of the AV so that individual jets could be isolated and collected. We used a 19 inch Colorado model AV. The inner latex bladder was thoroughly cleaned and disinfected using our standard protocol for reusable latex AV liners. The AV is filled with warm (50°C water and the bladder lubricated using a non-spermicidal lubricant. A sterile fingerless palpation sleeve is affixed to a wire loop with a handle to catch the sperm-rich fractions. The stallion mounts the phantom as usual and the collector places the AV on the penis. A second technician positions the collection device at the back of the open-ended AV to visualize the glans penis and the jets of semen as they are ejaculated. The collector places two fingers on the base of the penis to feel the urethra and alert the second technician as to when the jets were happening. The collector calls out the jets and after jets 1, 2, and 3 are collected the second technician closes off the collection bag and removes the device allowing for the remaining gel-contaminated jets to fall to the ground. For diagnostic purposes the remaining individual fractions can be collected by quickly exchanging a second collection bag/device after the sperm-rich fractions were collected. Using this technique, we could properly evaluate the semen from this stallion, prevent any gel contamination and successfully manage him in a cooled transported and frozen semen AI program.

Observation: poor initial sperm motility

1. Start by obtaining a thorough history on the stallion:
   Has he experienced a testicular insult or swelling of the scrotum? Is the stallion suffering from extreme heat stress or has he been in heavy training in a hot climate? Is there a possible history of steroid or other drug use? Has the stallion been ill or had a fever in the last two to three months?
2. Next, rule out the possibility of damage introduced during the collection process:
   Was the semen exposed to thermal stress? Was it trapped in a hot AV too long? Was the semen cold shocked from contact with cold collection vessel or exposure to cold ambient temperature? Was the semen contaminated with a non-isosmotic lubricant or excessive bacteria from dirty collection equipment? Did the stallion require several mounts before ejaculating? If so, was the collection vessel changed between mounts and/or was the pre-ejaculatory fluid removed. If not the semen is likely contaminated with debris and pre-ejaculatory fluid. Was the semen exposed to toxic residues on improperly rinsed collection equipment?
3. Rule out damage introduced during the initial semen handling and processing:
   Was the temperature of the water bath, incubator, extender, microscope slides and stage correct? Are the slides free of residues? (Even "pre-cleaned" slides often have a film on them than can affect sperm motility). Are you using a good quality extender to dilute the semen? Is it mixed properly? Did you check the motility of the raw semen? If it appears to be very active and the extended semen is very poor, you may have an issue with the extender.

If you suspect an issue with the extender, then it could be a sensitivity to the antibiotics in the extender or the type of extender. We have experienced stallions that have sensitivity to most milk based extenders and some with sensitivity to certain antibiotics. Try extending the semen on the next collection in a variety of different extenders with and without antibiotics and evaluate motility over time. We have also used complex freezing extenders which contain egg yolk and very little milk protein but without the cryoprotectant added to extend and chill semen from certain stallions that would not survive cooling using conventional extenders.

Once convinced that the poor motility was not induced by the collection process or analysis conditions there are a few diagnostic observations that can be made to try and better understand the cause of the poor motility.

1. Perform a morphology evaluation to determine if there are a high percentage of morphologically abnormal sperm in the ejaculate that could explain the poor motility.

2. Increase the collection frequency, perhaps even two collections an hour apart. Some stallions seem to accumulate sperm rapidly that deteriorate quickly with residence in the distal reproductive tract and exhibit much better motility when collected more frequently.

3. Some stallion's sperm are highly sensitive to exposure to their own seminal plasma. Collecting these stallions directly into extender placed inside the collection vessel may improve semen quality. In this situation, the sperm rich jets are diluted in the extender and somewhat protected from the toxic effects of the seminal plasma before the seminal plasma mixes with the sperm. You can also remove most of the seminal plasma by centrifuging the diluted semen, discarding the supernatant and replacing with extender for chilling semen. In cases of extreme seminal plasma toxicity, it may also be beneficial to collect fractionated ejaculates as described above.

4. Perform bacterial cultures on the semen to rule out a heavy bacterial load which can negatively impact sperm quality.

When these diagnostics do not provide an explanation of the poor initial sperm motility we advise the client to re-evaluate in two to three months to rule out a possible effect of season or transient poor semen quality due to some undetected illness or testicular insult that may have temporarily impacted spermatogenesis. If this is not an option, then we will suggest using a sperm selection technique such as single layer colloid centrifugation using EquiPure or Androcoll to separate the normal from abnormal sperm in the ejaculate and process only the enriched population. While this technique will result in an enriched population of good quality sperm, the recovery rate is limited by the number of functionally normal sperm in the ejaculate to begin with.

Observation: good motility but high volume, low concentration ejaculates

This type of ejaculate present a unique set of problems when freezing semen. We always dilute raw semen a minimum of 1:1 for centrifugation and when the sperm concentration is low (say <100 $\times 10^6$/ml), there may only be 2 $\times 10^9$ total sperm in each centrifuge tube. After aspirating the supernatant the volume of the sperm pellet and residual supernatant contributes significantly to the final volume of the extended semen even when the final desired concentration is only 200 $\times 10^6$/ml. The problem is even more pronounced when freezing at higher concentrations such as 400 x 10^6/ml as is standard in many laboratories. In these cases, adding extender that contains a concentration of cryoprotectant at 3-4% may result in the final extended sperm suspension having a cryoprotectant concentration as low as 1.5-2% which is likely too low to provide optimum protection for the sperm.

This is another reason why a high concentration, low volume ejaculate is desired for semen freezing and managing the stimulation of the stallion and the collection process to minimize seminal plasma is critical. In addition to managing the stallion to minimize gel production as discussed above, voiding the pre-ejaculatory fluid by deflecting the stallion's penis away from the AV after mounting and before inserting into the AV can significantly reduce the volume of the ejaculate resulting in a higher sperm concentration. Despite these measures some stallions will still produce ejaculates that have very low sperm concentration. In these instances one can adjust the final cryoprotectant concentration in the extended semen by adding supplemental cryoprotectant, either to the extender or to the extended semen after final dilution. This can be very difficult to do because the cryoprotectants are very viscous and difficult to accurately pipette in such small volumes.

Observation: decreased post-centrifugation motility

After centrifugation and resuspension in freezing extender, we remove an aliquot of the extended semen, dilute in the centrifugation extender, incubate 5 min and re-evaluate the motility on CASA. For most stallions, motility after centrifugation is similar to the fresh semen and sometimes better. However, occasionally the post-centrifugation motility is significantly reduced. In this case, first rule out any damage that could be related to improper analysis conditions or semen handling as discussed above. Next, try diluting another aliquot in freezing extender and re-evaluate. If the decrease is not seen when

diluted in freezing extender, there may be a problem related to cryoprotectant efflux from the cells following dilution in a cryoprotectant free extender. Further discussion on this point will follow.

On the next freeze try splitting the semen and diluting in different centrifugation extenders. Keep the original as one of the treatments as a control. If a cushioned centrifugation technique was used and the sperm pellets were difficult to re-suspend, try reducing the g-force or time. You can also try centrifugation without a cushion at lower g-force (300-350 x g, for 10-12 min). This technique will result in lower sperm recovery but may be less damaging to the sperm.

Observation: poor post-thaw motility

If the results of the initial freezing attempt are poor, efforts should be made to adjust the protocol to find a technique that may provide acceptable results for the client. We believe that there is a definite extender/protocol preference between stallions and this is a reason why we always perform split-ejaculate test freezes to evaluate different protocols on the initial attempts for new stallions. Dismissing a stallion as a poor candidate for a frozen semen program based on the negative results of a single test freeze using one standard protocol or extender type will eliminate several stallions that could be used successfully if efforts to customize the protocol had been employed.

Use of extenders containing lower molecular weight cryoprotectants such as ethylene glycol, propylene glycol and amides (methyl formamide and dimethyl formamide) alone or in combination can be beneficial for many stallions. Other variables in extender composition such as the types of sugars used, the source and amount of lipids and protein (milk and egg yolk) and the addition of buffers, chelating agents, and antioxidants can influence results. Also, the optimum rate at which sperm are cooled prior to freezing may be stallion or extender dependent.

One of the most significant stresses to sperm during freezing and thawing occurs after the sperm suspension is thawed. As mentioned above, when thawed sperm are placed in an extender that does not contain any cryoprotectant for post-thaw motility evaluation, the penetrating cryoprotectant diffuses out of the cell at a much slower rate than water moves into the cell to balance the osmotic gradient. This leads to an increase in cell volume and swelling of the plasma membrane that can be very damaging to sperm from some stallions. Differences in membrane composition (particularly the cholesterol to phospholipid ratio) between species and males within species may contribute to the ability of sperm from certain species and individuals to withstand the stresses of cryopreservation better than others. When a significant decrease is seen between pre-freeze and post-centrifugation or post-thaw motility for a stallion with good fresh semen quality, it may be a result of damage from this rapid increase in cell volume.

A way to test this theory is to evaluate motility of sperm diluted after thawing in extender containing cryoprotectant and compare with that of sperm diluted in extender without cryoprotectant. We do this by diluting sperm post-thaw to a concentration of 25 to 30 $x10^6$/ml in standard milk based centrifugation extender with or without cryoprotectant at the same concentration as the freezing extender. Because we have also seen instances of stallions whose semen does not survive well during post-thaw incubation in milk based extenders, we will also dilute aliquots of the thawed semen in freezing extender and freezing extender that does not contain cryoprotectant. If the motility is better in the extenders containing cryoprotectant, we suspect a sensitivity to cryoprotectant efflux. If the motility is better in the freezing extenders (with or without cryoprotectant) than in the milk based extenders (with or without cryoprotectant) then we suspect an issue with incubation in milk based extenders and will perform centrifugation and post-thaw motility evaluation using cryoprotectant-free freezing extender.

If the issue is cryoprotectant efflux one can perform a serial dilution of the cryoprotectant by exposing the thawed sperm to extender with increasingly lower concentrations of cryoprotectant resulting in a series of several smaller volume changes over time as opposed to a single large volume change. If this protocol results in a better retention of sperm motility, one can implement a similar post-thaw treatment to sperm prior to insemination since presumably the same issue occurs when thawed sperm are exposed to cryoprotectant-free uterine fluids following insemination.

When discussing the relative "freezability" of individual stallions it is important to consider the decrease in any measure of semen quality relative to the value for the unfrozen fresh sample. All

cryopreservation protocols will damage some sperm from all stallions and the change in quality between pre-freeze and post-thaw samples is a measure of how good sperm from that stallion/ejaculate survived the process. A stallion (stallion A) that has a pre-freeze progressive motility of 70% and a post-thaw progressive motility of 35% is very different than one (stallion B) that had a pre-freeze progressive motility of 45% and a post-thaw value of 35%. Even though the post-thaw motility and the number of progressively motile sperm are the same for each stallion and meet the suggested minimum standards for commercial use, sperm from stallion B appears to be more resistant to damage from cryopreservation than stallion A. Furthermore, one may suspect that because of the significant drop in motility for stallion A, the sperm that remain progressively motile may have also suffered sub-lethal damage that could impact longevity in the female reproductive tract and fertility. This is another reason to strive to obtain the best possible quality for each individual stallion by customizing protocols to minimize the damage to sperm during cryopreservation.

I have referenced motility as the primary assay for measuring sperm quality throughout this manuscript but it is important to remember that motility is only one functional attribute of sperm required for fertilization. However, objective, accurate and repeatable measures of motility still seem to be the best and most practical single assay of relative cell health that can be readily performed on all batches of frozen semen. Additional assays of sperm function such as flow cytometric measures of sperm membrane integrity, mitochondrial function and DNA integrity in combination with motility may provide additional value to determine the potential fertility of a given sample of frozen semen. These assays should be considered for stallions whose fertility with frozen semen is poor even though post-thaw motility is good.

One should also consider whether the defective sperm in the dose are suffering from a defect that is compensable by increasing the total number of sperm in the insemination dose. If the damaged sperm do not compete with the fully functional sperm for fertilization, then acceptable fertility may be achieved by increasing the number of total sperm in the insemination dose. Rectally guided, low dose insemination in situations where the number of functional sperm are reduced may also increase fertility and lastly, the technique of intracytoplasmic injection ICSI provides an option for achieving pregnancies from even the poorest quality frozen semen.

Effect of ovulation synchronization with or without progesterone supplementation and pubertal status of beef heifers on reproductive performance

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Physiological diversities of beef heifers at the time of breeding offer both challenges and opportunities to implement cost effective synchronization protocols. The objective was to determine the interaction effect of ovulation synchronization with or without progesterone (P4) supplementation and pubertal status of beef heifers on reproductive performances. The hypothesis was pubertal heifers with endogenous P4, and pre- and peri-pubertal heifers that received exogenous P4 will attain higher pregnancy rate (PR) to fixed time AI (FTAI).

Angus cross beef heifers (n=1821) from eight locations were assigned a body condition score (BCS; 1 to 9; 1, emaciated; 9, obese), a reproductive tract score (RTS; 1 to 5; 1, immature RT, acyclic; 5, mature RT, cyclic) and a temperament score (1, calm and walk; 2, excited and jump, trot or run) at enrollment. Within locations heifers were assigned to different synchronization protocols based on their reproductive tract score. Heifers with RTS 2 to 4 (pre- and peri-pubertal) were assigned to either CO-Synch (n=442; TFCS group) or CO-Synch+CIDR (n=437; TFCCS group) protocols. Similarly heifers with RTS 5 (pubertal) were assigned to either CO-Synch (n=468; FCS) or CO-Synch+CIDR (n=474; FCCS) protocols. Briefly, On Day 0, heifers in all groups were given GnRH (100 µg i.m., Cystorelin) and heifers in TFCCS and FCCS groups were administered a controlled internal drug release (CIDR; 1.9 g P4; Eazi-Breed CIDR) vaginal insert. Seven days later, CIDRs were removed from heifers in TFCCS and FCCS groups and PGF2α (25 mg i.m., Lutalyse) was given to all heifers. Estrus detection aids were applied to all heifers at CIDR removal. Heifers were inseminated at 72 h after CIDR removal, and second dose of GnRH was administered concurrently. At insemination, estrus status of all heifers was recorded as positive (YES) or negative (NO). Clean-up bulls were introduced to heifers 14 d after FTAI and remained with the heifers until the end of 85 d breeding season. Pregnancy diagnosis was performed by ultrasound at d 60 after FTAI. Data were analyzed with logistic regression with random intercepts using GLIMMIX procedures of SAS (version 9.4). Accounting for BCS (<5, 49.4 [309/626] vs. ≥ 5, 59.8% [715/1195]; $P<0.0001$), heifers that expressed estrus or not (Yes, 60.2 [638/1060] vs. No, 50.7% [386/761]; $P<0.0001$), and temperament score (calm, 58.5 [650/1105] vs. excited, 52.2 [374/716]; $P<0.01$), the AI-PR (Table) differed among 4 groups. The heifers in TFCCS and FCCS groups had greater AI-PR compared to heifers in TFCS group. The AIPR for heifers in FCS group, and heifers in TFCCS and FCCS groups were not different.

Groups	CIDR	Pubertal status	n	AI-PR
TFCS	No	Pre- and peripubertal	442	52.3[a]
FCS	No	Pubertal	468	54.7[ab]
TFCCS	Yes	Pre- and peripubertal	437	58.8[b]
FCCS	Yes	Pubertal	474	59.2[b]

[ab]different superscripts within column were significant ($P<0.05$)

In conclusion, the AI-PR following the use of CO-Synch protocol in pubertal beef heifers did not differ from the AI-PR of all beef heifers following the use of CO-Synch+CIDR protocol. The use of CO-Synch protocol in pubertal heifers eliminated the cost for CIDR and still resulted in an acceptable AI-PR. It is feasible that the differences in pubertal status of beef heifers at breeding can be exploited by submitting those heifers to different cost-effective estrous synchronization protocols.

Keywords: Beef heifers, pubertal status, ovulation synchronization, artificial insemination, pregnancy rate.

Effect of seminal plasma and equilibration time on post-thaw quality of bovine semen

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The long-term goal of the consortium is to optimize an on-farm semen freezing protocol. The use of electroejaculation for on-farm semen collection yields ejaculates with variable amounts of seminal plasma and variable response to cryopreservation. In addition, a prolonged equilibration time may allow for transport of semen to the laboratory and further processing the day after collection. It was hypothesized that removing seminal plasma by centrifugation improved post-thaw quality of semen collected with electroejaculation, and extending the equilibration time to 24 h did not have a detrimental effect on post-thaw semen quality. One ejaculate was collected from nine adult beef bulls using electroejaculation. Each ejaculate was divided into four aliquots: NC5: non-centrifuged, equilibrated for 5 h; NC24: non-centrifuged, equilibrated for 24 h; CE5: centrifuged, equilibrated for 5 h; CE24: centrifuged, equilibrated for 24 h. All aliquots were extended to 50 x 10^6/ml in OptiXcell (IMV Technologies, Maple Grove, MN). Non-centrifuged aliquots were refrigerated immediately after dilution. Centrifuged aliquots were centrifuged at 600 x g for 10 min, and the pellet was re-suspended to 50 x 10^6/ml. Semen was then equilibrated at 5°C for 5 or 24 h. Then, semen was loaded into 0.5-ml straws and frozen following a standard protocol. Sperm motility was evaluated on frozen-thawed semen using computer assisted semen analysis, and membrane integrity (propidium iodide), acrosome integrity (peanut agglutinin) and apoptosis (annexin V) were evaluated using fluorescence activated flow cytometry. Variables were compared among the four treatment groups using ANOVA. The percentage of total motile (P=0.951), progressively motile (P=0.897), membrane-intact (P=0.077) or apoptotic spermatozoa (P=0.805) did not differ among treatments (Table). However, centrifugation resulted in a decrease in the percentage of acrosome-intact spermatozoa ($^{a,b}P<0.0001$; Table). Under the conditions of this study, extending the equilibration time had no effect on the parameters evaluated. Removal of seminal plasma by centrifugation had no beneficial effect on sperm motility, membrane integrity or apoptosis. However, centrifugation had a negative effect on acrosome integrity. Therefore, centrifugation of bovine semen extended in Optixcell at 600 x g for 10 min is not recommended for cryopreservation.

Table: Post-thaw quality of non-centrifuged or centrifuged semen equilibrated for 5 or 24h.

Variable	NC5	CE5	NC24	CE24
Total motility (%)	39.7 ± 5.7	35.9 ± 5.3	41.4 ± 8.2	39.1 ± 7.4
Progressive motility (%)	37.5 ± 5.7	32.4 ± 5.4	38.7 ± 8	33.7 ± 7.7
Apoptotic cells (%)	53.5 ± 7.6	56.9 ± 5.3	49.8 ± 6	50.6 ± 2.2
Membrane intact cells (%)	40.2 ± 6.9	49.9 ± 6.2	55.6 ± 7.5	65.1 ± 5.8
Acrosome intact cells (%)	90.3 ± 4.2^b	40.9 ± 7.7^a	95.1 ± 2.4^b	59.3 ± 7.3^a

Keywords: Bovine, bull, semen, cryopreservation, centrifugation

Association of bovine sperm WW domain-binding protein with capacitation status and head morphology

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Sperm-borne oocyte-activating factors are critical for oocyte activation. One protein that is potentially involved in oocyte activation is postacrosomal WW domain-binding protein (PAWP). Postacrosomal WW domain-binding protein is an evolutionarily conserved protein in mammals, found exclusively in the perinuclear theca of the sperm head postacrosomal sheath. In human and bovine spermatozoa, high PAWP expression is positively associated with artificial insemination, IVF and ICSI fertilization rates. We hypothesized that sperm PAWP content is associated with sperm head morphology and independent of acrosome status. Our objectives were to analyze PAWP content for spermatozoa with normal and abnormal head morphology, and under conditions conducive for no capacitation (NC), capacitation (C) and inducing the acrosome-reaction (AR). Frozen semen samples from four fertile bulls were analyzed in duplicate. Sperm samples were thawed, washed and incubated for 40 min at 37°C in one of three media formulated to induce: 1) no capacitation, S-TALP without $NaHCO_3$ or BSA; 2) capacitation, S-TALP with 25 mM $NaHCO_3$, 6 mg/ml BSA and 60 µg/ml heparin sodium; or 3) acrosome reaction, capacitating medium with 5 µM calcium ionophore A23187. For immunofluorescence, samples after incubation were washed, fixed, permeabilized, blocked and incubated with affinity-purified rabbit anti-PAWP antiserum as the primary antibody, following by goat anti-rabbit Alexa-488 as the secondary antibody (Molecular Probes™, Eugene, OR). For DNA detection and acrosome evaluation, spermatozoa were stained with 1 µg/ml Hoechst 33258 and 1 µg/ml PNA-TRITC (Sigma Aldrich, St. Louis, MO). PAWP content, acrosome status, and sperm head morphology (normal or abnormal) were evaluated in individual spermatozoa (n=100 per bull and replicate) by immunofluorescence and differential interference contrast microscopy. Analyses of variance, least squares means, and odds ratios, with 95% confidence interval, were performed to determine treatment differences. Four patterns of PAWP intensity were assigned: optimal (normal), moderate, low and null. Among NC, C and AR treatments, no differences were observed for PAWP intensity and acrosome status. Within NC, some spermatozoa had undergone the acrosome reaction; optimal PAWP was observed in more spermatozoa with intact vs reacted acrosomes (39.0±14.6 and 19.5±3, $P=0.04$). In C and AR, fewer spermatozoa with intact acrosomes had low or no PAWP than spermatozoa with reacted acrosomes (C, 3.0±5.4 and 13.3±2.1; AR, 5.5±3.9 and 0.25±0.5; $P<0.03$). The percentages of spermatozoa with normal head morphology were higher ($P<0.05$) when PAWP intensity was optimal than for all other intensity groups. The proportion of spermatozoa with optimal and moderate PAWP increased with higher percentages of normal head morphology, independent of capacitation conditions; which significantly increased the probability of spermatozoa with desired PAWP content (optimal, 2.1 OR, CI 1.4-3.1 and moderate, 1.6 OR, CI 1.1-2.6). In conclusion, conditions that induce sperm capacitation or acrosome reaction did not affect relative PAWP content. However, the presence of head defects was negatively associated with PAWP content in bull spermatozoa, suggesting that spermatozoa with abnormal head morphology may have lower potential to induce oocyte activation.

Serum amyloid A concentrations and correlations with sepsis scores in newborn foals born from mares with experimentally with experimentally induced placentitis

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Placentitis is an important cause of pregnancy loss and neonatal death in horses. Foals born from mares with placentitis are typically septic and premature. Despite numerous developments in recent years in the literature concerning mares with experimentally induced placentitis, there have been few developments in regards to treatment and diagnosis of sepsis in foals born from mares with placentitis. Serum amyloid A (SAA), a major acute phase protein, was recently described to be a useful diagnostic and prognostic marker for mares with experimentally induced placentitis, and to be increased in serum of fetuses from placentitis mares. Therefore, we hypothesized that SAA is increased in the plasma of newborn foals born from mares with experimentally induced ascending placentitis, and that SAA is correlated with sepsis score. The objective of this study was to describe SAA concentrations and its association with sepsis score in newborn foals born from mares with induced ascending placentitis. Thirty-four foals enrolled in the study were divided into three groups according to sepsis score and placentitis as follows: healthy foals (control group, n=6), placentitis/non-septic foals (non-septic group, n=19) and placentitis/septic foals (septic group, n=9). Sepsis score was performed as described elsewhere.[1] Survival rates were assessed until 30 days of life. Septic foals were treated with ampicillin, flunixin and fluid therapy as needed during the first seven days after delivery. Blood samples were obtained at foaling, 24 and 48 hours after delivery for white-blood cell counts, fibrinogen, lactate, and SAA concentrations. Continuous data were analyzed by ANOVA-RM, and categorical data by Fisher's exact test. Results were expressed as mean ± SEM and significance was set at $p<0.05$. All foals of control group were classified as non-septic. The rate of survival was lower in the group of septic foals (22%, n=2/9; $p<0.001$) than the non-septic group (89%, n=17/19) and control group foals (100%, n=6/6). The concentration of SAA was significantly lower in healthy control foals and non-septic foals than septic foals: at birth (0.64±0.30mg/dl; 0.71±0.28 mg/dl; 211.03±102.18 mg/dl), 24h (62.60±33.94 mg/dl; 34.5±10.52 mg/dl; 241.86±132.03 mg/dl) and 48h of life (88.95±65.42 mg/dl; 106.31±58.14 mg/dl; 457.73±205.86 mg/dl). There were no significant differences in lactate, leukocyte counts, or fibrinogen for foals at birth. At 24h and 48h of life, the plasma concentration of lactate was significantly higher in foals from the septic group than healthy controls and non-septic groups. It is worth noting that fibrinogen and leukocyte counts were not significantly different between all three groups at 24 and 48 hours after delivery. In conclusion, SAA is a sensitive marker for sepsis in newborn foals from mares with experimentally induced ascending placentitis. Conversely, fibrinogen and leukocyte counts were not useful markers for sepsis in this population of foals. Lactate did not appear to be a useful screening foal at delivery, as it was not different between groups, but was increased at 24 and 48 hours after foaling.

Keywords: Sepsis, markers, biochemical, metabolism, survival

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pH from mammary gland secretions is acidic at the time of parturition but remains neutral during the first week postpartum in mares

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Serial assessment of pH from mammary gland secretions is an inexpensive and reliable method to determine impending parturition in mares. Most mares carrying normal pregnancies foal with a secretion pH of 6.2-6.6, and electrolyte concentrations and pH are significantly correlated. However, previous studies have demonstrated that some mares foal before showing a drop in pH and increase in calcium, and it is unclear if these mares show these changes at the time of parturition. Additionally, mammary gland secretion pH could change minutes after collection due to changes in temperature, increase in bacteria, and oxygenation. The objectives of this study were: (i) to determine pH and electrolyte concentrations from mammary gland secretions collected prepartum and at the time of parturition, (ii) to characterize milk pH in the first week postpartum, and (iii) to evaluate pre-foaling mammary secretions pH at three storage temperatures. We hypothesized that (i) mammary gland secretions present an acidic pH, increased calcium, magnesium, and potasium and decreased sodium at time of foaling, regardless of prepartum pH and electrolyte concentrations, and (ii) pre-foaling secretion pH varies with storage temperature and time in an initial value dependent manner. Light breed mares (n=25) carrying normal pregnancies presented for foaling management were examined daily for signs of impending parturition, and small aliquots (0.5-2 mL) of pre-foaling mammary gland secretions were collected twice a day until foaling. Secretion pH was measured with a portable pH meter (Compact pH METER B-71X, HORIBA Scientific, LAQUAtwin, Japan), and aliquots were preserved at -20°C for electrolyte evaluation. Calcium (Ca^{2+}), sodium (Na^{+}), potassium (K^{+}) and magnesium (Mg^{2+}) concentrations were measured using an automated Analyzer (AU 480 Beckman Coulter, CH 1260 Nyon, Switzerland) for the seven days pre-foaling. Eighteen 5ml aliquots were harvested to determine the effect of temperature and time on secretion pH. Samples were divided into three groups based on pH: 8 (range 7.8-8.2), 7.5 (range 7.3-7.7), 7 (6.7-7.2), and 6.5 (6.2-6.6). Immediately after collection, samples were equally divided into three storage conditions: 37°C, 21°C, and 5°C. Secretion pH was measured at 0, 15, 30, 45 and 60 min, and then repeated hourly for 10 h after collection. Milk samples were collected from all mares to evaluate pH once daily for seven days after parturition. All data analyses used JMP 12.1 (SAS Institute Cary, NC, USA). Concentrations of electrolytes and pH were analysed using mixed models for seven days pre-foaling. When significant, post hoc comparisons were made with Tukey's LSD test. Two ways interactions were examined between fixed and continuous effects in the different models. Results of milk pH for the first week postpartum were compared with a mixed model. The significance is set as $p \leq 0.05$. All the data are expressed as mean ± SEM. All mares had high Ca^{2+}, Mg^{2+}, K^{+}, and low Na+ at the time of parturition. All three mares with an alkaline pH up until parturition had an acidic pH at the time of foaling. The pH of mammary gland secretions was slightly acidic (6.8-6.9) the first two days postpartum, but became neutral (7-7.1) for the next five days. Storage temperature of mammary gland secretions did not significantly affect pH up to 45 minutes of storage. Longer storage did result in significant variation in pH. In conclusion, mares with an alkaline pH, low Ca2+, and high Na+, did change pH and electrolyte profile before foaling but changed so rapidly that twice a day sampling may miss the change. Mare milk pH became neutral by three days postpartum. Storage temperature of mammary gland secretions did not affect pH for 45 min.

Keywords: Foaling, horses, mammary gland secretions storage

A comparison of the effects of carbon dioxide and medical air for abdominal insufflation on respiratory parameters in sheep undergoing sedated laparoscopic artificial insemination

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Laparoscopic artificial insemination (LAI) has better conception rates and utilizes semen more efficiently when compared to conventional trans-cervical approach and can be performed under light sedation in a hospital or field setting. The most commonly used insufflation gas is carbon dioxide (CO_2) because it rapidly diffuses into blood and can be expelled via the lungs, but filtered air has been suggested as a safe and economical alternative. Our goal was to evaluate the effects of these two insufflation gases on respiratory parameters such as partial pressure of arterial oxygen (PaO_2), partial pressure of arterial carbon dioxide ($PaCO_2$), and arterial blood pH. Since air does not readily diffuse into the blood stream, we hypothesized that abdominal insufflation with medical air will have improved respiratory parameters when compared to insufflation with CO_2 in sheep undergoing sedated LAI procedure.

Each animal underwent estrus synchronization for timed artificial insemination two weeks prior to, and fasted for at least 24 hs before the day of the procedure. Thirty-four sheep were randomly selected and enrolled in the study with owner permission. Each animal was assigned a participation number, which was randomized to one of two treatment groups: CO_2 or medical air. The ear was clipped, sterilely prepared, and local anesthetic was applied to aid in placement of a 20 gauge arterial catheter, which was then utilized for serial arterial blood gas sampling. Blood samples were obtained at baseline (T0), after sedation (T1), two minutes after being placed in Trendelenburg position (T2), five minutes post-abdominal insufflation (T3), and 15 minutes after the procedure was completed and patient had been returned to a standing position (T4). Arterial blood gas samples were collected in heparinized syringes, stored on ice, and batch run within 1 to 2 hs of collection. These samples were analyzed on a Stat Profile pHOx Ultra blood gas analyzer.

A t-test showed that there was no statistical difference between the two insufflation gases when comparing changes in $PaCO_2$, PaO_2, and pH at pre- and post-insufflation times points ($P>0.005$). Values for $PaCO_2$, PaO_2, and pH that may result in clinically significant consequences were defined prior to data collection. These were defined as an increase in $PaCO_2 >10\%$, a decrease in $PaO_2 >10\%$, or a decrease in pH $>5\%$. There was also no difference between CO_2 and medical air in the number of sheep who experienced clinically significant changes.

There appears to be no statistical or clinically significant difference between CO_2 and medical air as insufflation gases, when evaluating their effects on oxygenation, ventilation, and acid-base parameters in sheep undergoing sedated LAI. Medical air may be a comparable and economical alternative to the commonly used CO_2, especially if it is easily available with the use of a filtration system. It is worth noting that 30% of the sheep in both groups became clinically hypoxemic ($PaO_2<70$ mm hg) and supplemental oxygen via facemask may be warranted during LAI procedures. None of the study participants in either group developed any post-surgical complications such as peritonitis or signs of systemic or local infection following the procedure.

Keywords: Laparoscopic artificial insemination, sheep, carbon dioxide, medical air, blood gas

Serum and placental oxytocinase in healthy late pregnant and postpartum mares

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Oxytocinase (OTase)/insulin regulated aminopeptidase (IRAP) or leucyl-cystinyl aminopeptidase (LNPEP) varies in pregnant women with higher levels in the third than the first trimester of pregnancy. Oxytocinase is present in the chorionic microvilli of human placenta, hydrolyzes several peptides including oxytocin and vasopressin, and is suggested to be responsible for maintaining pregnancy homeostasis. There is paucity of information on the activity and presence of OTase in placental tissues and serum of pregnant mares. The objectives of this study were: 1) characterize serum OTase of pregnant mares in the last month of gestation and post-partum; and 2) characterize the effect of location on OTase expression in placental tissues of mares. Jugular blood samples were taken from 18 Standardbred mares: (a) prepartum (320-336 days of gestation), (b) 24 hours before parturition, (c) 20 minutes and (d) 2 hours after foaling. Serum was stored frozen until analysis. Chorioallantoic membrane tissues were retrieved from the body (B), pregnant horn (PH), and non-pregnant horn (NPH) immediately after placental delivery in (n=8) mares, then divided and stored frozen at -80°C, or 10% formalin. Tissue samples (100 mg) were thawed, rinsed and homogenized in phosphate buffered saline using sonication and then centrifuged at 1500 x g for 15 minutes. The resulting supernatant was stored at -80°C until assayed. A commercial ELISA (LNPEP for horses, MYBioSource, San Diego, CA) with a detection range of 6.25-200 U/L and an intra and interassay coefficient of variation <15%, which was validated in our laboratory, was used. Immunohistochemical (IHC) staining for LNPEP with negative and positive controls (Rabbit anti-LNPEP, Thermo Fisher Scientific, Rockford, IL) using an automated slide strainer (Autostainer Plus, Dako Canada Inc., Mississauga, ON) was conducted. Proprietary software (STATA/SE version 13.1, College Station, TX) using $p<0.05$ was used including: Shapiro-Wilk for normality, ANOVA and Kruskal Wallis to evaluate the effect of Day and Placental Region on OTase. Post-hoc analysis was performed using Dunn's Test. Mare's mean gestational age was 342±7.1 days (range 332 to 361 days). There was no significant effect of Day on serum OTase levels. The OTase levels (U/L; mean±SD) at sampling times were: (a) (41.34±10.80), (b) (42.74±13.38), (c) (40.36±15.68), and (d) (38.78±13.50). There was a significant effect of Placental Region on OTase ($p= 0.0058$), with body and pregnant horn ($p=0.0098$ and $p=0.001$, respectively) having significantly higher levels than non-pregnant horn. The placenta OTase activity U/gr [median (quartiles)] by placental region were: B (29.55 [15.49, 36.47]), NPH (14.65 [11.74, 17.40]), and PH (28.88 [21.14, 36.88]). Evaluation of IHC showed strong staining of OTase in the PH. This is the first description of the presence of OTase in equine placenta. Further investigation is required to determine if serum and placental OTase activity varies in healthy or abnormal equine pregnancies, or if levels are associated with retained fetal membranes.

Keywords: Oxytocinase, late term pregnancy, placenta, serum

Effects of acute and chronic infusion of kisspeptin on luteinizing hormone and follicle stimulating hormone in prepubertal bulls

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Kisspeptin (KP) is a hypothalamic neuropeptide that stimulates the secretion of gonadotropin releasing hormone. The aim of the present study was to determine the effects of acute and chronic infusions of KP on the serum concentrations of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in prepubertal bull calves. The hypothesis was that chronic infusion of kisspeptin would result in prolonged increased concentrations of LH and FSH. Holstein bull calves (n = 16; 12±1 wks; 96.5±14.5 kg) were treated with one of four doses of KP (KP-10; human Metastin 45-54; 0 [control], 0.125 [low], 0.25 [medium], or 0.5 ug/kg/hr [high])by intravenous infusion for 76 hs. Blood samples were collected every 15 min for the first (acute; 1 to 6 hs) and last (chronic; 71 to 76 hs) 6 hs of the infusion to determine the serum concentrations and pulse parameters of LH and FSH. The statistical significance of LH and FSH concentrations was analyzed using a mixed affects repeated measures analyses of variance. Whereas acute infusion of KP-10 increased ($P < 0.05$) mean LH concentrations and the number of pulses ($P < 0.05$), chronic infusion had no effect. Nadir of LH concentrations was greatest following infusion of the medium and high doses KP-10 ($P < 0.05$) and during the acute period ($P < 0.05$). Mean FSH concentrations were greatest during the acute infusion period ($P < 0.05$) and least during the chronic infusion period with medium and high doses of KP-10 ($P < 0.05$). Number of FSH pulses was greatest during the acute infusion period ($P < 0.05$). Amplitude of FSH pulses was greatest during the acute infusion period ($P < 0.05$) and least during the chronic infusion period ($P < 0.05$). There was no effect ($P > 0.05$) on the nadir of FSH concentrations. In conclusion, acute infusion of KP-10 increased LH concentrations and pulse parameters, and chronic infusion of KP-10 decreased FSH concentrations and pulse parameters. Despite the potential suppression of the hypothalamic-pituitary-gonadal axis with chronic infusion of KP-10, there may still be potential applications of kisspeptin, kisspeptin analogs, or kisspeptin receptor agonists to hasten the onset of puberty in livestock.

Keywords: Kisspeptin, chronic infusion, bull, puberty

Pharmacokinetics of oral micronized progesterone and intravaginal progesterone administration in the bitch

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The aim of this study was to determine the pharmacokinetics (PK) of vaginally (Crionone®, Serono Laboratories, Norwell, MA) and orally delivered micronized progesterone (Prometrium®, Solvay Pharmaceuticals, Inc., Marietta, GA) in the bitch. We hypothesized that both vaginal and oral treatments would result in a dose-dependent increase in concentrations of plasma progesterone. We further hypothesized that oral dosing of micronized progesterone would result in greater, sustained plasma progesterone than those recorded in bitches treated with intravaginal (IVA) micronized progesterone gel. Eight adult sexually intact bitches in anestrus were arranged in a 4x4 Latin square with repeated measures experimental design. Each subject rotated through four different progesterone treatment groups with a minimum seven day-wash out period between treatments: 100 mg oral micronized progesterone, 200 mg oral micronized progesterone, 45 mg intravaginal micronized progesterone and 90 mg intravaginal micronized progesterone. Blood samples from each subject were obtained at time points 0, 0.5, 2, 1.5, 2, 4, 6, 8, 12, 24, 36, 48 and 72 hs following treatments. Concentrations of plasma progesterone were determined by RIA (ImmuChem Double Antibody, 125I RIA Kit, MP Biomedicals, Costa Mesa, CA). Pharmacokinetic analysis was carried out using commercially available software (Phoenix WinNonlin 6.4, Certara Inc., Princeton, NJ). One-compartmental (intravaginal) and non-compartmental (oral administration) modeling were performed to analyze data using the mean concentrations for each dosing to calculate the area under the curve (AUC), maximum plasma concentration (C_{max}), time elapsed to reach C_{max} (T_{max}), and elimination half-life ($t_{1/2}$).

Results for the 100 mg and 200 mg oral doses and 45 mg and 90 mg IVA doses were as follows: AUC, 30.86, 187.96, 90.64, and 226.68 $ng.h.ml^{-1}$, respectively; Cmax, 13.47, 169, 8.68, and 13.24 $ng.ml^{-1}$, respectively; Tmax, 0.5, 0.5, 0.84, and 1.67 hs, respectively, and half-life, 5.87, 6.76, 6.6, and 10.65 hs, respectively.

Micronized progesterone was readily absorbed in bitches when administered either orally or intravaginally. Contrary to our initial hypothesis, the extent of body exposure to progesterone as indicated by the area under the curve was greater when intravaginal micronized progesterone was used. The ability of intravaginal preparations of micronized progesterone to induce sustained progesterone exposure may provide an alternative strategy for treating pregnant dogs whenever hypoluteoidism is being suspected.

Keywords: Micronized progesterone, pharmacokinetics, canine

Use of hypertonic extender to cryopreserve sauger (*Sander canadensis*) spermatozoa

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Freshwater fish species typically exhibit poor sperm quality following cryopreservation. In anadromous species, cryopreservation extenders hypertonic to the seminal plasma have been used successfully to improve post-thaw sperm quality and fertilization rates. The objective of this study was to determine the effect of extender osmolality on post-thaw sperm quality in an economically valuable freshwater fish, the sauger (*Sander canadensis*). We hypothesized that extenders hypertonic to the seminal plasma would enhance dehydration during cryopreservation resulting in increased post-thaw sperm quality. Fresh milt from male saugers (n=10) was diluted to $1x10^9$ sperm/mL in cryopreservation extenders with osmolalities of 350 (isosmotic), 500, or 750 mOsm/kg (E350, E500 and E750, respectively) and equilibrated for 10 min at 5°C. Samples were then diluted 1:1 (v:v) with the respective extender containing 20% dimethyl sulfoxide as cryoprotectant and equilibrated prior to cryopreservation. Sperm quality was assessed prior to cryoprotectant addition, prior to cryopreservation and after thawing. Sperm motility parameters were assessed by computer-assisted sperm analysis (CASA) and sperm viability was determined by fluorescence microscopy using a LIVE/DEAD® Sperm Viability Kit (Molecular Probes Inc., Eugene, OR). Data were evaluated by repeated measures ANOVA with Fisher's LSD test. Significance was set at $P<0.05$. Total and progressive motility were significantly lower in E750 after cryoprotectant addition (26.25±4.96% and 6.88±2.43%, respectively) compared to E350 (82.15±3.10% and 24.28±2.79%, respectively) and E500 (77.50±4.54% and 27.03±4.35%, respectively). Spermatozoa extended in E350 and E500 had higher velocities (97.76±8.42 and 100.53±8.63 μm/s, respectively) compared to E750 (71.71±9.63 μm/s) after cryoprotectant addition. Extender osmolality had a significant effect on all post-thaw sperm quality parameters. Extender E500 yielded the highest post-thaw progressive motility (32.20±1.20%) and velocity (84.97±5.32 μm/s) compared to E350 (19.50±1.50% and 62.60±5.07 μm/s, respectively). Spermatozoa in extenders E350 and E500 displayed the highest total motility (65.30±1.40% and 68.70±2.00%) and viability (80.60±1.50% and 78.80±1.20%), respectively. Moreover, E750 yielded the lowest sperm velocity (38.17±2.11 μm/s), viability (71.80±1.20%), total motility (12.10±1.60%) and progressive motility (1.60±0.60%) post-thaw. In conclusion, the use of a hypertonic extender with osmolality of 500 mOsm/kg resulted in higher sperm velocity and progressive motility post-thaw compared to an isosmotic extender. However, cryosurvival was poor when an extender with osmolality of 750 mOsm/kg was used, indicating that the upper osmotic tolerance limit of sauger sperm lies between 500 and 750 mOsm/kg. The improvements in sperm cryosurvival obtained in our study lay the foundation for future experiments evaluating the fertilizing capacity of freshwater fish sperm cryopreserved in hypertonic extenders.

Keywords: Spermatozoa, sauger, osmolality, hypertonic, cryopreservation, extender

Exogenous nerve growth factor-β improves corpus luteum function and enhances conceptus development in cattle

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Nerve growth factor-β (NGF) has been identified in the seminal plasma of domestic mammals and is important for ovulation induction in camelids. While previously thought to play a negligible role in spontaneous ovulators, recent studies have suggested that it may have a luteotrophic effect in heifers. The objective of the current study was to determine if systemic administration of NGF, purified from bovine seminal plasma, would improve corpus luteum (CL) function and enhance conceptus development. Our hypothesis was that systemic administration of NGF to cows at time of artificial insemination (AI) would lead to increased CL size, progesterone (P4) production, pregnancy-specific protein B (PSPB) concentrations, and expression of interferon-stimulated genes (ISGs), markers of conceptus development and maternal recognition of pregnancy. Purification of NGF was performed from bull seminal plasma using a combination of anion and cation exchange chromatography and gradient elution. Beef cows were randomly assigned to CONT (n = 30) or NGF (n = 30) groups and synchronized using a 7-day Co-Synch + CIDR program. At time of AI (d 0), NGF cows received 296 μg purified NGF, reconstituted in 12 mL phosphate buffered saline (PBS), and CONT cows received 12 mL PBS intramuscularly. Blood samples were collected from the coccygeal vein of each cow for quantification of plasma concentrations of P4 (d 0, 3, 7, 10, 14, 19) and PSPB (d 24). Peripheral blood leukocytes were harvested at d 19 for measuring expression of ISGs (ISG15, MX1, MX2, RTP4) by qPCR. Transrectal ultrasound was also performed for determination of ovarian structures and CL volume (d 0-19). Pregnancy diagnosis was performed by transrectal ultrasound at d 28. Statistical analysis was performed using analysis of variance with repeated measures (SAS 9.4, Cary NC). There were no treatment differences in the presence ($p = 0.96$) or size ($p = 0.96$) of dominant follicles at d 0 or in the occurrence of greater concentrations of P4 at AI (> 1.0 ng/mL; $p = 0.99$) or ovulation ($p = 0.93$) before enrollment. Corpus luteum volume increased over time ($p < 0.001$), but did not differ between treatment groups ($p = 0.46$). NGF cows had increased peripheral P4 over CONT cows from d 10-19 ($p = 0.04$). Pregnancy rates at d 28 were 75% in NGF cows versus 59% in CONT cows ($p = 0.13$). In pregnant cows, PSPB concentrations at d 24 were greater in NGF than CONT cows ($p < 0.05$). Additionally, fold-change expression of ISG15 and MX2 at d 19 were greater in pregnant NGF cows than in pregnant CONT cows ($p < 0.05$), but no differences for MX1 and RTP4 were present. Collectively, these results demonstrate that NGF administration at AI improved CL function and enhanced ISG expression and PSPB concentrations. Future studies are warranted to investigate whether NGF can be used to improve reproductive efficiency of cattle.

Keywords: Bovine, development, luteotrophic, ovulation-inducing factor, progesterone

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Evaluation of biofilm production by *Escherichia coli* isolated from clinical cases of canine pyometra

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Many strains of *Escherichia coli* (*E. coli*) have the ability to produce biofilm, a matrix of extracellular polymeric substances that confers antimicrobial resistance, leading to increased morbidity and recurrent infections. In dogs, production of biofilm by *E. coli* has been observed in bacteria isolated from recurrent cases of urinary tract infections. However, to date, studies addressing biofilm presence during canine uterine infections caused by *E. coli* are lacking. The objective of this study was to determine the role of biofilm production by *E. coli* during uterine infection by: 1) confirming the presence of biofilm *in situ,* and 2) determining the ability of different strains of *E. coli* to produce biofilm *in vitro*. We hypothesized that most strains of *E. coli* involved in canine pyometra will be capable of producing biofilm both *in vivo* and *in vitro*. Samples used in this study were obtained during ovariohysterectomy of dogs affected by pyometra (n=13). A swab of the uterine contents was collected immediately after surgery and submitted for aerobic culture. Two sections of the uterine horns were preserved in 3% glutaraldehyde and 10% buffered formalin. At approximately 24 h of culture, isolated bacteria were identified by matrix assisted laser desorption/ionization and *E.coli* isolates were frozen at -80°C. Only tissue samples from cases confirmed to have *E.coli* infection were evaluated by scanning electron microscopy (SEM), histopathology and biofilm assay. During SEM, the surface of the endometrium was evaluated for the presence of bacteria and/or a fibrous matrix. Sections submitted for histopathology were stained with hematoxylin and eosin and periodic acid-Schiff (PAS) and evaluated by a board certified veterinary pathologist blinded to treatments. *E. coli* isolates were analyzed for biofilm formation by microtiter biofilm assay using crystal violet. Optical densities were compared by analysis of variance (ANOVA), using StatPlus software. Significance was set at $P<0.05$. Nine out of thirteen cases (69%) resulted in pure growth of one or two *E.coli* colonies, totaling 11 different isolates. Areas suggestive of the presence of biofilm were observed on all samples on SEM; however, bacteria consistent with *E. coli* were only visualized in three samples. All tissues exhibited endometrial inflammation with varying degrees of luminal exudate on histopathology. Visible bacteria were observed in five specimens. Mucus was located within cystic endometrial glands and occasionally overlying epithelium in seven specimens. Nine isolates (9/11, 82%) had significantly higher optical densities then negative controls, indicating *in vitro* biofilm production. In conclusion, we demonstrated that clinically relevant strains of *E. coli* produce biofilm both *in vivo* and *in vitro,* supporting our hypothesis. Development of new treatment modalities for pyometra aimed at disrupting biofilm may enhance therapeutic efficacy allowing for preservation of the reproductive potential of genetically valuable dogs.

Keywords: *Escherichia coli*, biofilm, canine, pyometra

Concentrations of sulfadiazine-trimethoprim in serum and endometrium of mares treated with an oral suspension

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We hypothesized that a novel oral suspension of potentiated sulfonamide would reach adequate tissue concentrations greater than the in vitro MIC reported for common uterine pathogens. The objective of our experiment was to assess the concentrations of sulfadiazine-trimethoprim in plasma and the endometrium in non-pregnant mares following treatment with an oral formulation.

To test our hypothesis, twenty healthy cycling mares, (ages 3 to 18 y; median 10.5 y) had endometrial biopsies performed and were declared free of endometrial inflammation per histology. In a subsequent estrus, transrectal ultrasonography was performed on the mares to determine the presence of uterine edema and a follicle ≥30 mm in diameter. These mares were treated (0 h) with a suspension of sulfadiazine-trimethoprim (333 mg/67 mg combination per mL; Equisul-SDT®, Aurora Pharmaceuticals, LLC, Northfield, MN) at a dosage of 24 mg/kg administered PO (nasogastric gavage) every 12 h for five treatments. Blood samples were obtained at 0 h, 12 h, 36 h and 60 h. An endometrial biopsy was also performed at 60 h and endometrial samples were snap-frozen in liquid nitrogen. Drug concentrations in the endometrial tissue were determined by liquid chromatography. A Pearson product-moment correlation test was used to measure the strength of association of the relative concentrations of antimicrobials in the plasma and endometrium.

Concentrations of plasma antibiotics increased with time during treatment. Mean (±SEM) concentrations of plasma sulfadiazine were 5.17±0.34, 10.22±0.64 and 13.39±0.71 µg/mL and of trimethoprim 0.038±0.01, 0.15±0.03 and 0.27±0.04 µg/mL at 12, 36 and 60 h, respectively. Endometrial concentrations of sulfadiazine and trimethoprim at 60 h were 7.96±0.47 µg/g and 0.23±0.03 µg/g, respectively. The correlation coefficients between plasma and endometrial tissue concentration of sulfadiazine and trimethoprim were R^2=0.81 and R^2=0.94 ($p<0.0001$), respectively.

Sulfadiazine-trimethoprim concentrations achieved in the endometrium after five consecutive treatments with the oral suspension were above the *in vitro* MIC reported for common pathogens known to cause bacterial endometritis, e.g., *Streptococcus zooepidemicus* (MIC= 0.25 to 4 µg/mL) and *Escherichia coli* (>0.25 to 4 µg/mL). The oral suspension of sulfa-trimethoprim should be an efficacious and viable treatment for bacterial endometritis.

Keywords: bacterial endometritis, sulfadiazine, trimethoprim, antibiotic tissue penetration, endometrium.

Reproductive parameters of white-tailed deer (*Odocoileus virginianus*) bucks

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White-tailed deer (WTD) farming is an expanding industry in the United States, leading to an increased demand for improving breeding practices in captive cervids. While studies in other cervid species have shown superior semen quality at peak breeding season, there is limited information regarding breeding soundness parameters for WTD bucks. The objective of this study was to establish reproductive parameters for WTD early (September, SEPT) and at peak rut season (December, DEC). We hypothesized that reproductive parameters would improve at the peak of the breeding season. In SEPT and DEC 2016, mature WTD bucks (n=11 and 8, mean age of 2.6 ± 0.3 y, range 2.5-3.5 y) were remotely anesthetized with tiletamine-zolazepam (0.4 mg/lb) and xylazine (1 mg/lb) intramuscularly. All bucks were in hard antler at the time of first sample collection (SEPT), and two bucks were sold and one was euthanized between SEPT and DEC collections. Semen was collected by electroejaculation and evaluated for total sperm ejaculated and morphology. Computer-assisted semen analysis was used to immediately assess sperm motility. Scrotal circumference (SC) was measured using tape, and testicular length (TL), width (TW), and height (TH) were measured with ultrasound. Transrectal ultrasound (linear 5 MHz transducer) was used to measure the length (L) and width (W) of the bulbourethral (BBG) and vesicular (VG) glands. Data were analyzed using two-sample t-test and Wilcoxon rank sum test in R. Data are expressed as mean ± SEM and ranges. Semen output had a tendency to be higher in DEC (2±0.6 billion, range: 0.65-6.39) than in SEPT (1±0.2 billion, range: 0.22-2.08; p=0.08). Percent of total (TM%) and progressive (PM%) sperm motility were increased in DEC (TM%: 80±8.0, range: 21-95; PM%: 75±8.7, range: 11-91) as compared to SEPT (TM%: 71±4.4, range: 34-86; PM%: 58±5.0, range: 29-81; $p \leq 0.01$). The percentage of morphologically normal sperm increased in DEC (85±4.8 %, range: 58-96%) vs. SEPT (63±7.8%, range: 4-93%; p=0.05), with fewer primary defects observed in DEC (8.5±3.8%) than in SEPT (27±8.9%; p=0.03). There were no significant differences for secondary defects between SEPT (11±2.4%, range: 0-23%) and DEC (6.6±3.1%, range 0-29%; p=0.18) collections. Interestingly, proximal droplets were the most common primary defect recorded in SEPT (16.4±8.5%, range: 1-95%), whereas no animals presented this defect in DEC. In SEPT, two bucks produced semen with remarkable asthenozoospermia and teratozoospermia; one of these bucks had normozoospermia in DEC, but the second buck was euthanized before DEC collection. Surprisingly, SC was larger in SEPT (19.2±0.5 cm, range: 17-23 cm) than in DEC (17±0.3 cm, range: 15.5-18.5 cm; $p<0.01$). Measurements of TL, TW, and TH were symmetrical ($p \geq 0.7$), with a tendency for increased TL (3.7±0.2 cm) and TW (3.3±0.1 cm) in SEPT vs. DEC (TL 3.0±0.05 cm; TW 2.7±0.03 cm; $p \leq 0.07$). The BBG were symmetrical in L and W ($p \geq 0.7$) with no differences between SEPT (L 2.5±0.09 cm; W 1.3±0.01 cm) and DEC (L 2.4±0.08; W 1.3±0.07 cm; $p \geq 0.7$). The VG were symmetrical in L and W ($p \geq 0.7$), with a tendency for L to be increased in DEC (2.8±0.1 cm) vs. SEPT (2.4±0.1 cm; p=0.07). Collectively, these results supported our hypothesis that reproductive parameters improved at peak rut. However, despite semen quality being inferior early in the breeding season for most bucks, the semen quality appeared to be suitable for cryopreservation or fresh insemination, though with a reduced yield.

Keywords: Accessory sex glands, andrology, cervid, semen analysis

Holstein heifer behavior in self-locking stanchion at fence-line mangers and its impact on reproductive performance

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The utilization of self-locking stanchions is widespread where large dairy herds ise prevalent. Acts of aggression were elevated during all periods following restraint, but the use of self-locking stanchions did not appear to affect overall wellbeing of the cattle. However, their behavior while locked in the stanchion and its effect on reproductive performance has not been described and must be further explored. The objective of the study was to determine impact of heifer behavior during head lock restraint at feeding on the reproductive performances. The hypothesis was aggressive escape behavior will result in reduced reproductive performance.

Holstein heifers (n=817) from four farms (stocking density: 0.96, 1.00, 0.89, 1.10) were evaluated for their behavior during headlock restraint in the self-locking stanchion at fence-line mangers at the time of feeding. Heifers were evaluated from the alleyway approximately for 15 minutes after they were locked. They were assigned a score 2, if they expressed aggressive escape behavior (pulling backward multiple times with banging) or 1, if they expressed mild escape behavior (pulling backward without banging) or 0, calm in absence of escape behavior. All heifers were assigned a BCS (1, emaciated to 5, obese). The heifers were followed for three inseminations to determine the impact of behavior on the first service pregnancy per AI (FSP/AI) and cumulative P/AI (CP/AI) from three services. Age of heifers and pregnancy information were retrieved from records. Data were analyzed using PROC ANOVA and PROC GLIMMIX (SAS version 9.4).

The heifers with aggressive, mild and calm escape behaviors were 26.7, 28.2 and 45.2%, respectively ($P<0.05$). The stocking density did not alter behavior ($P>0.1$). Overall the escape behavior did not affect general health and welfare.

Accounting for BCS ($P<0.05$), the first service pregnancy/AI was different for heifers that exhibited calm, mild or aggressive escape behavior: 58.0 (214/369), 53.5 (123/230) and 48.2% (105/218), respectively ($P<0.05$). There was no difference in FSP/AI between heifers with mild and aggressive behaviors ($P>0.1$); however, a trend for differences in FSP/AI between calm and mild behaviors was observed ($P<0.1$). The FSP/AI for heifers with BCS <2.5, 2.5 to 3.5 and >3.5 were 44.2% (34/77), 56.5% (341/604) and 50.7% (69/136), respectively ($P<0.05$). The age of the heifers did not influence the FSP/AI ($P>0.1$). Three was no interaction for FSP/AI between age of the heifers and escape behavior, and BCS category and escape behavior ($P>0.1$). Accounting for BCS ($P<0.0001$), the CP/AI was different for heifers that exhibited calm, mild and aggressive escape behavior: 84.8% (313/369), 71.3% (164/230) and 64.7% (141/218), respectively ($P<0.0001$). There was a difference in CP/AI between heifers with mild and aggressive behaviors ($P<0.0001$); however, no difference was observed between calm and mild behaviors ($P>0.1$). The CP/AI for heifers with BCS <2.5, 2.5 to 3.5 and >3.5 were 61.0 (47/77), 80.5% (486/604) and 62.5% (59/83), respectively ($P<0.0001$). The age of the heifers did not influence the CP/AI. There was no interaction for CP/AI between age of the heifers by lock-up behavior and BCS category by lock-up behavior ($P>0.1$).

In conclusion, aggressive and mild escape behaviors during headlock restraint at feeding showed a negative effect on reproductive performance of dairy heifers by lowering their P/AI.

Keywords: Holstein heifer, behavior, self-lock stanchion, artificial insemination, pregnancy

Effect of two anthelmintic agents on parasitic load, body condition and reproductive performances of beef cows

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An appropriate parasite-control program in the cow herd can protect against economic losses by preventing substantial production losses. The objective of the study was to compare the effect of two anthelmintic agents, pour-on and extended-release injectable parasiticides, on parasite load, body condition and reproductive performances of beef cows. The hypothesis was beef cows that receive extended-release injectable parasiticide will have reduced parasite load, improved body condition, and better reproductive performance over their pour on counterparts.

Four weeks prior to the beginning of the breeding season, beef cows (n=966) from six locations were assigned a body condition score (BCS). Within locations cows were randomly assigned to CON and TRT groups. Cows in CON group received pour-on (CON; Ivermectin) and cows in TRT group received extended-release injectable parasiticides (TRT; Eprinomectin). Fecal samples were collected from all cows prior to treatment. All cows were synchronized with CIDR + CO-Synch protocol and inseminated once. Two weeks later cows were grouped with clean-up bulls (at 1:40 cow to bull ratio) for the reminder of the 85 d breeding season. Fecal samples were collected again at 60 d after AI (90 d after the treatment) at the time of pregnancy diagnosis by ultrasonography. The cows were re-examined for pregnancy 30 days after the end of the breeding season. Fecal eggs were counted within two days of sampling by two clinicians using McMaster method and fecal egg per gram (EPG) was calculated. For both treatment groups, the cows were categorized as responders (R) and non-responders (NR) based on reduction in fecal EPG from pre- to post-treatment (R=100% reduction in EPG). Further BCS was assigned to all cows at the fall pregnancy diagnosis to determine BCS difference (BCSD); lost (LO) vs maintained or gained (MG) categories) between pretreatment and at fall pregnancy diagnosis.

The mean EPG for responders in CON and TRT groups were -42.8 and -85.7. The mean EPG for non-responders in CON and TRT group were 132.8 and 32.9. The percentages of non-responders were significantly different between CON and TRT groups (22.6 vs. 7.7%, respectively; P=0.05; Chi-Square test). The outcome measured were BCSD, AI pregnancy (AIPR) and breeding season pregnancy rates (BSPR; PROC GLIMMIX of SAS). The mean AIPR between TRT (66.1%; 328/496) and CON group (61.3%; 288/470) were not different (P=0.12). There was an interaction between treatment groups and response categories for the AIPR. The AIPR were 63.2 (230/363), 54.7 (58/106), 66.8 (306/458) and 57.9% (22/38) for CON-R, CON-NR, TRT-R, and TRT-NR, respectively (P<0.05). The BSPR of CON and TRT groups were 90.2 and 93.1%, respectively (P=0.1). The BSPR were not different among response categories (P>0.1), but were significantly different among BCSD categories. A greater percentage of cows lost BCS in CON than in TRT group, 24.0 (113/470) vs. 11.3% (56/496), respectively (P<0.0001), and a greater percentage of cows maintained or gained BCS in TRT than in CON group, 8.7 (440/497) vs. 76.0% (357/470), respectively (P<0.0001). Among cows in MG category, the BSPR was different between CON and TRT group, 87.1 (311/357) vs 94.1% (414/440), respectively (P<0.001). Among cows in LO category, there was no difference in BSPR between CON and TRT group, 832. (94/113) vs 85.7 (48/56), respectively (P>0.1).

In conclusion, eprinomectin was better than ivermectin in reducing parasite load and improving BC. The AIPR was greater for cows that responded to eprinomectin than cows that did not respond to ivermectin. Among cows that maintained or gained BCS, the BSPR was greater for the cows that received eprinomectin than cows that received ivermectin.

Keywords; Beef cows, paraciticide, body condition, pregnancy rate

Hypertrophic osteopathy in a castrated dog with prostatic carcinoma and prostatitis

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A 9-year-old, castrated male Border Collie cross-breed dog first presented to our clinic with fever and lumbar pain. Hematology and biochemistry were not diagnostic and the dog was treated empirically at the owner's request. Three weeks later the dog developed painful, warm swellings in all distal limbs and radiographs of the distal limbs were consistent with hypertrophic osteopathy. There were no visible chest lesions detected on radiographs. An enlarged prostate was detected on radiographic and ultrasonographic examination. The prostate was cavitated on ultrasonography. The owner was reluctant to pursue further diagnostics.

The dog continued to deteriorate and was euthanized 40 days after presentation due to signs associated with the hypertrophic osteopathy. Post-mortem examination revealed that the patient had a prostatic carcinoma with severe chronic suppurative prostatitis.

Hypertrophic osteopathy with only abdominal masses without an obvious metastasis is uncommon and has not been reported before in a castrated dog with prostatic carcinoma. There is another report of hypertrophic osteopathy associated with prostatic neoplasia in an intact dog without pulmonary metastases.[1]

Interestingly, in both this case and the other reported case, the tumor was associated with bacterial prostatitis. It is possible that this combination may increase the production of cytokines which lead to development of hypertrophic osteopathy.

Prostatic neoplasia should be considered along with abdominal neoplasia in dogs presenting with hypertrophic osteopathy where no thoracic lesions are found. In addition, we suggest that dogs with both prostatic carcinoma and prostatitis should be treated aggressively for prostatitis when giving palliative care in case this combination leads to hypertrophic osteopathy.

Keywords: Dog, castrated, prostate, carcinoma, hypertrophic osteopathy

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The canine vaginal microbiome and associations with puppy survival

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Perinatal and neonatal deaths account for a significant financial and emotional loss to breeders each year. Bacterial infection during parturition such as from vaginal group B Streptococcus in humans is associated with increased risk of neonatal septicemia but this has not been evaluated in canines.

Objectives

1) To describe the vaginal microbiome during pregnancy, and in postmortem samples of stillborn and neonatal loss puppies. 2) To determine if there are associations between vaginal microbiome during pregnancy, and likelihood of litters with stillbirths and neonatal puppy losses.

Hypothesis

We hypothesize that (1) the bacterial diversity of the canine vagina is greater than previously thought, (2) that novel bacteria not previously associated with stillbirths are identified, and (3) that there are differences in the vaginal microbiome between bitches that have normal, healthy puppies and puppies that fail to thrive.

Study design

Vaginal samples during the last week of pregnancy were evaluated using metagenomic analysis. Bacterial DNA was extracted and the 16S ribosomal RNA gene amplified by PCR for sequencing. The 16S ribosomal RNA gene was used to identify the bacterial genus of origin. Differences between bacterial populations in the vagina of animals that have high stillbirth rate were evaluated. Samples of liver, and stomach contents from stillborn puppies were assessed using metagenomic analysis and compared with the vaginal sample of the dam to determine if there is an association between the maternal microbiome and stillbirths. Discriminant analysis using JMP Pro 12 was used to evaluate the correlation between the bacterial taxa in the different samples. Prevalence of bacteria in each sample was used as a covariate in a stepwise discriminant analysis model. Variables were removed in a stepwise manner until only variable with a $P < 0.05$ were retained in the final model.

Results

The five most common bacterial genera in the vagina regardless of stillbirths and puppy health conditions are *Bradyrhizobium, Streptococcus, Gemella, Mycoplasma,* and *Avibacterium.* The five most abundant genera in the microbiome of the stillborn puppy liver samples are *Bradyrhizobium, Serratia, Phenylobacterium, Enterococcus,* and *Flavobacterium.* The most abundant five genera in the stomach content samples of the stillborn puppies are *Streptococcus, Acinetobacter, Bradyrhizobium, Enterococcus,* and *Serratia.* The presence of the genera *Biberstinia, Staphylococcus, Pasteurella, Corynebacterium* and *Methylobacterium* in the vagina of the dam all significantly increased the probability that there would be a stillborn puppy in the litter. However, none of these bacteria are found in significant levels in the stillborn puppy liver or stomach content samples according to discriminant analysis.

Discussion/conclusion

We identified five genera that are correlated with incidence of stillbirth, but further studies are required to determine bacterial causation of stillbirth. However, if the presence of specific bacteria in the vagina is associated with puppy outcomes, treatment options such as antimicrobials may be able to reduce puppy losses and diseases.

Keywords: Canine, microbiome, stillbirths, neonatal losses

Cross-species comparison of chromosomal instability during mammalian pre- implantation development

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One of the first major milestones in early mammalian development is blastocyst formation and yet, less than half of pre-implantation embryos from most mammals will reach this stage following *in vitro* fertilization (IVF). A leading cause of IVF failure and embryo loss in humans is the presence of whole chromosomal imbalances, or aneuploidy. Although more likely to arrest at the cleavage-stage, aneuploid embryos may still form blastocysts and often morphologically indistinguishable from chromosomally normal (euploid) embryos. Chromosomal mis-segregation in oocytes during meiosis is considered the primary reason for aneuploidy in cases of advanced maternal age. However, mosaic aneuploidies, which are mitotically derived, occur just as frequently and irrespective of maternal age. The potential cause(s) of mitotic aneuploidy, whether it can be non-invasively detected, and if pre-implantation embryos from other mammalian species are also chromosomally unstable was the focus of this study. Using a combination of time-lapse imaging to monitor embryo development, immunofluorescent analysis of nuclear structure, and next generation RNA-Sequencing (RNA-Seq), we assessed mitotic divisions in rhesus macaque (N=54), equine (N=11), bovine (N=48), and mouse embryos (N=45) up to the blastocyst stage. While similar mitotic timing was observed between rhesus, equine and bovine embryos, mouse embryos exhibited a particularly long second division of ~19.8±3.2 hours ($p<0.0001$), which is likely due to species-specific differences in the onset of embryonic genome activation. Upon immunostaining with the nuclear envelope marker, LAMIN-B1, only intact primary nuclei were detected in cleavage-stage mouse embryos, whereas chromosome-containing micronuclei were observed in rhesus, equine, and bovine embryos in order of decreasing frequency. Besides persisting up to the blastocyst stage, these micronuclei were often positive for gamma-H2AX, a marker of DNA breaks and chromosome fragility. RNA-Seq analysis of rhesus, equine, and bovine blastocysts showed high expression of ribosomal proteins, cytochrome oxidases, and housekeeping genes as somewhat expected. Interestingly, exceedingly high expression levels (RPKM≥2,400) of ANNEXIN A2 (ANXA2), a gene known to be involved in diverse cellular processes, was detected in blastocysts of all three species. Given recent findings that ANXA2 participates in cytokinesis and is associated with chromosomal instability in other cell systems, its abundant and conserved expression may be important for maintaining embryo ploidy status during pre-implantation development across higher-order mammalian species.

Keywords: Aneuploidy, imaging, micronuclei, mitosis, sequencing

The use of bipolar clamps for neutering in small animal practice

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Ovariectomy (OE), ovariohysterectomy (OHE) and castration are the most common methods of contraception in dogs and cats. Therefore, they are the most commonly performed surgical procedures in small animal practice in many countries. However, OE and OHE in mature, large bitches can be difficult and time-consuming due to the challenge of hemostasis. The aim of this study was to determine whether the use of bipolar clamps in OE and OHE of bitches and queens and in castration in dogs improved the ease and reduced the time of surgery.

Bipolar clamps were used to assist with hemostasis in OE and OHE in bitches and queens and for castration in dogs. The bipolar clamps initially used were 6 inches and 8 inches (Peebee Endoscopy, www.peebeeindia.com) and subsequently 6.5 inches and 7.6 inches (Baisheng Medical Company, www.obs-medical.com). There were 1,321 bitches spayed, 818 queens spayed and 1,154 dogs castrated during the time of this study from September 2011 to August 2016.

In bitches, all veterinary surgeons using the clamp to obtain hemostasis had the opinion that OE was easier and faster than suturing. Statistical analysis by a linear regression model demonstrated that the use of bipolar clamps reduced surgery time in bitches. The model also revealed clear effects of the age and weight of bitches being related to surgery time. There was also an effect of experience of veterinary surgeon being inversely related to surgery time.

In queen OE and dog castrations, surgery time was not greatly reduced nor was the ease of surgery greatly improved by using bipolar clamps.

Post-operative hemorrhage was not detected in any animal operated on. A complication initially encountered was superficial minor burns to the skin after the use of cautery. Protection of the skin by placement of a gauze swab between the skin and the clamp prevented this. It could be more difficult to obtain hemostasis in bitches with a large uterus such as those in diestrus and sometimes a uterine ligature was necessary to supplement the cautery. In cases of OHE in pyometra and cesarean spays, transfixion ligature was also necessary on the vaginal stump. There were some technical problems on occasion resulting from malfunction of the clamp or the cable.

Bipolar clamps are safe and effective and their use reduced the time of OE in bitches.

Keywords: Cat, dog, bipolar cautery, castration, ovariectomy

Ovarian cyst, mastitis, pyometra, and cardiac disease in a German Shepherd bitch

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An 8 month old German Shepherd (GS) bitch was evaluated at the University of Florida Veterinary Medical Teaching Hospital (UFVMTH) for a prolonged first estrous cycle of nine weeks duration. Previously, at 5 months of age, the bitch was diagnosed at the UFVMTH with a potentially lethal GS inherited ventricular cardiac arrhythmia. Mexiletine 150 mg, po, TID, and sotalol (ß blocker) 40 mg, po, BID, were administered in an effort to manage the arrhythmia. Upon presentation, the dog was active, bright, alert, and responsive. The arrhythmia was not evident. Mammary glands were small, non-lactating, and palpably normal. Moderate serosanguineous discharge from an enlarged turgid vulva was present. Vaginal epithelial cytology showed complete cornification, the presence of red blood cells, bacteria, and no evidence of white blood cells. *Brucella canis* serology was negative and serum progesterone (P4) was <0.2 ng/ml. Abdominal ultrasonography revealed within the left ovary, a 3.4 x 2.4cm thin walled anechoic structure, consistent with a follicular cyst, while the right ovary was normal. The uterus did not contain luminal fluid, but was thickened, suggesting hyperplasia. Gonadotrophin releasing hormone, 45 microg, im, was administered once daily for four days. Additionally, human chorionic gonadotropin, 1000 Units, im, was administered on days one and three of treatments. Eleven days later, the bitch was re-examined due to severe focal mastitis with spontaneous abscess rupture of the right caudal gland. Slight serous discharge from a moderately enlarged vulva was present. Cornification was again complete, and neutrophils were not observed. Progesterone was 2.9 ng/ml, and the left ovarian cyst was 3.2 x 2.9 cm, with echogenic content. Specimens for aerobic culture were obtained from the ruptured gland, and cefovecin 232 mg, im, was administered. One week later, the mastitis had resolved, the vulvar discharge had ceased, the vaginal cytology was no longer cornified, contained non-degenerate neutrophils, and rare metestrum cells, indicating diestrus, and serum P4 was 4.9 ng/ml. Six weeks later the bitch suffered an open pyometra and emergency ovariohysterectomy was performed at the UFVMTH with uneventful recovery; arrhythmia was not present. Six months later, at seventeen months of age, the dog presented for fever, lethargy, and vomiting. A 4 cm intra-abdominal abscess was identified on ultrasonography and aspiration cytology and exploratory laparotomy recommended. The owners elected humane euthanasia.

Keywords: Ovarian cyst, mastitis, pyometra, abscess, cardiac arrhythmia

Normal and neoplastic canine lymphocytes express luteinizing hormone receptors

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Introduction

Lymphoma is a common malignant cancer of dogs. Spayed and castrated dogs are 3-4 times more likely to develop lymphoma.[1,2] Circulating luteinizing hormone (LH) concentrations are significantly and persistently elevated in spayed and castrated dogs compared to intact dogs. Luteinizing hormone receptors (LHR) are expressed in normal rat lymphatic tissue.[3]

Hypothesis

That LHR were also present in normal and neoplastic canine lymph nodes. The study aim was to determine if LHR were expressed and to quantify the level of expression.

Methods

Normal (n=1) and neoplastic (n=4) lymph node tissue was removed postmortem, formalin-fixed, paraffin-embedded, and sectioned (6 μm) onto charged slides. Testicular tissue from a separate dog obtained following castration was treated in the same manner for a positive control. All slides were deparaffinized, rehydrated, subjected to heat-induced epitope retrieval (#S1700, Dako, Carpinteria, CA). Endogenous peroxidase activity was inactivated with 3% H_2O_2 and nonspecific binding was blocked with 1% horse serum. Goat polyclonal anti-human LHR antibody (SC-26341, Santa Cruz Biotechnology, Dallas, TX,) was applied at a 1:50 dilution. Negative controls from each tissue were treated in the same way except in absence of primary antibody. Slides were reacted with biotinylated horse anti-goat IgG (Vector Laboratories, Burlingame, CA) and incubated with preformed avidin-biotin-peroxidase complex (#PK6105, ABC kit, Vector Laboratories) followed by Nova Red Peroxidase substrate (#SK4800, Vector Laboratories). Slides were counter-stained with hematoxylin, dehydrated, and mounted. The percentage of cells positive for LHR was determined at 400X magnification by a single observer.

Results

Canine lymphocytes express LHR in 4% of cells in normal lymph nodes and 12.37% of the cells in those with lymphoma based on a single observer counting the cells (Figure). There was no positive staining evident in the negative control tissue sections.

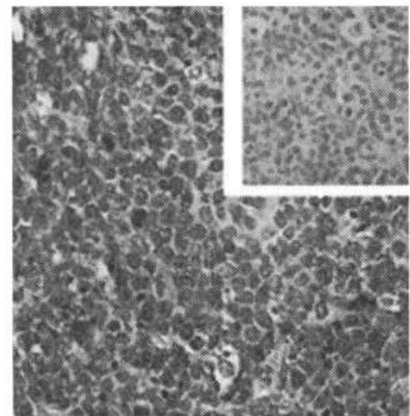

Figure. LHR expression in a 7-yr-old mixed breed spayed female dog with lymphoma. Negative control in upper right.

Discussion

This is the first report of LHR expression in canine lymphatic tissue. Studies are underway to determine the immunophenotype (B- or T-) of the lymphocytes expressing the LHR. The long-range goal of this research is to provide evidence to support using a complementary treatment for canine lymphoma by down-regulating LH with a commercially-available canine gonadotropin-releasing hormone (GnRH) agonist.

Keywords Castrate, dog, lymphoma, spay

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Comparison of serum estradiol, progesterone and luteinizing hormone concentrations, follicular development and timing of ovulation in dairy heifers treated with 4- or 5-day CoSynch+CIDR protocols

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The use of 5-day CoSynch+CIDR protocols in dairy heifers has resulted in pregnancy per timed AI (P/TAI) ranging from 52.2 to 61%. A previous study evaluating a Monday to Friday 4-day CoSynch+CIDR protocol in dairy heifers showed an adequate P/TAI (55.0%) which was not statistically different from that in the 5-day CoSynch+CIDR (63.3%). However, there was a tendency of higher (8.3%) P/TAI in the 5-day group, which might be associated with differences in follicular development and timing of ovulation between groups. We hypothesized that heifers treated with 4-day CoSynch+CIDR protocol have longer interval from CIDR removal to ovulation and different levels of estradiol (E_2) and luteinizing hormone (LH) compared to heifers treated with a 5-day CoSynch+CIDR protocol. The objectives were to compare follicular growth and timing of ovulation (interval from CIDR removal to ovulation) in dairy heifers treated with 4- or 5-day CoSynch+CIDR, using ultrasonography (USG) per rectum and determine the serum progesterone (P_4), E_2, and LH levels in the same heifers using radio-immunoassay (RIA). Twelve cycling Holstein heifers (12-15 mo), were randomly assigned to either the 4- or 5-day Co-Synch+CIDR groups (n=6/group) to receive an intravaginal Eazi-Breed CIDR® insert containing 1.38 g of P_4 for 4 or 5 days, respectively. At CIDR removal, 25 mg of PGF2α (Lutalyse®) was injected intramuscularly (IM); 72h after CIDR removal, heifers received 100 µg of GnRH (Factrel®) IM and AI with commercial frozen-thawed semen. Follicular growth and timing of ovulation were assessed using ultrasound per rectum every 12h on the first day and every 6h on the subsequent three days after CIDR removal. Blood samples were collected at initiation of protocols for determination of P_4, and at TAI for determination of P_4 and E_2, and every 6h during the first and second day after CIDR removal and every two hours during the third day after CIDR removal, to assess LH levels. Descriptive statistics were calculated for timing of ovulation, follicular diameter and hormone concentrations (mean ± SD). Heifers in the 4-day group had smaller follicles at the time of CIDR removal, TAI and before ovulation (7.2±2.8, 12.0±1.2, and 12.3±1.4 mm) compared to heifers in the 5-day group (10.7±2.7, 13.1±2.8, and 14.0±2.0 mm). Five out of 6 heifers (83.3%) in the 4-day group ovulated at 90-96h after CIDR removal, while most heifers in the 5-day group (4/6; 66.6%) ovulated at 84-90h after CIDR withdrawal. Progesterone concentration at TAI was <1 ng/mL in all the heifers. Serum E_2 concentration at TAI was higher in the 4-day group than the 5-day group (3.7±2.7 vs 0.8±0.6 pg/mL). Heifers in the 5-day group reached higher LH levels (10.01 ± 5.8 ng/mL) than heifers in the 4-day group (6.6 ± 2.7 ng/mL) during the sampling period. In conclusion, heifers in the 4-day group had smaller follicular diameter, longer interval from CIDR removal to ovulation (6h longer), higher concentrations of E_2 at TAI, and lower LH levels during the evaluation period than heifers in the 5-day group. These results support our hypothesis and suggest that prolonging the interval from CIDR removal to TAI by 6 hours (from 72 to 78h) in heifers treated with 4-day CoSynch+CIDR would increase P/TAI.

Keywords: Co-Synch+CIDR, heifer, LH, estradiol, timing of ovulation

A 5 ml dose PG-600® is detrimental to ovarian function and pregnancy rate in ewes during the breeding season

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PG-600® (Intervet/Merck Animal Health, Madison, NJ) is a single dose injectable product containing equine chorionic gonadotropin (80 IU/ml) and human chorionic gonadotropin (40 IU/ml), which is labeled for estrus induction in swine. This drug is also routinely used off-label for out-of-season estrus induction in sheep. However, at the most common dose administered to ewes (3-5 ml), PG-600® is likely to overstimulate the ovaries, resulting in reduced pregnancy rates. The aim of the current study was to determine if a lower dose of PG-600® would not reduce the pregnancy rate. We hypothesized that the pregnancy rates of ewes treated with a low dose of PG-600® during the breeding season would not be different from controls. Polypay ewes were treated with CIDR inserts (Eazi-Breed™ CIDR, Zoetis, Kalamazoo, MI) for 10 days. Two days prior to CIDR removal, cloprostenol (125 µg; Estrumate®, Intervet/Merck Animal Health) was given intramuscularly. On the day of CIDR removal (day 0), ewes were divided randomly into three groups (n=8) to receive 5 ml PG-600® (T1), 1.5 ml PG-600® (T2), and 5 ml saline (C). Jugular vein samples were collected prior to the PG-600® injection (0 hour) and at 2, 4, 8, 12, 24, 48, 72, 96, 120, 168 and 336 hours after injection. Serum estradiol-17β and progesterone were determined by chemilluminescence (Immulite 1000, Siemens Healthcare Diagnostics, Tarrytown, NY). Following PG-600® injection, ewes were rotated every eight hours between pens containing a new fertile ram for four days. Ewes were examined via transrectal ultrasonography from 9-11 days after PG-600® injection to count the number of corpora lutea (CLs) and via transabdominal ultrasonography 35 and 63 days after PG-600® for pregnancy diagnosis. An analysis of variance (ANOVA) was used to compare the average number of CLs and pregnancy rate between treatments. Repeated measures ANOVA was used to determine the effect of treatment on hormone concentrations. Significance was defined as $p<0.05$. Serum progesterone concentrations were increased during the ensuing 336 hours after injection in T1 compared to C and T2. Although there was a significant effect of time, there was no effect of treatment on serum estradiol-17β concentrations when compared over 336 hours after injection. The average number of CLs was greater in the T1 ewes (3.2) compared to C (2.2) and T2 ewes (2.3). Pregnancy rate was also lower in the T1 ewes (37%) compared to C (75%) and T2 (87.5%). These data suggest that the 5 ml dose of PG-600® administered to ewes during the breeding season alters ovarian function and either impairs fertilization or reduces embryo survival. This experiment will be repeated with the same ewes out of the breeding season to determine if the 1.5 ml dose of PG-600® will be as effective as the 5 ml dose in inducing estrus and if the lower dose will result in higher pregnancy rates.

Keywords: Corpus luteum, estradiol, estrus induction, progesterone, sheep

Acknowledgement

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Evaluation of diagnostics for ante-mortem testing of ovarian follicular dysplasia (OFD) in cattle

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The objectives of this study were to more thoroughly evaluate the presence of ovarian follicular dysplasia (OFD) in Florida herds, assess characteristics of OFD positive cattle through ultrasound examination, endocrine profiles, and RNA analysis in order to develop an ante-mortem test for reliable identification of OFD in cattle. The RNA sequencing and gene analysis was performed by Hudson-Alpha Institute for Biotechnology. Four hundred and fifty cull cows selected by private veterinarians representing five Florida ranches received reproductive tract palpation, ultrasound examination and blood collection. Based on the ultrasound findings, 66 total cows (10-16 per ranch) were followed to slaughter the following day for collection of reproductive tracts, and ovarian sampling. Five non-OFD and five OFD were selected for RNA sequencing. Ovaries with OFD were graded histologically I to IV and follicular morphometrics were recorded. Circulating serum levels of progesterone (P4) and anti-Mullerian hormone (AMH) were quantified in 200 cows. Ovarian follicular dysplasia was diagnosed in 57.6% of cows followed to slaughter. Infertility from other diseases was diagnosed in 12.1% of these cows and 30.3% were determined to be normal. The distribution of OFD grades was; Grade I: 44.7%, Grade II: 39.5%, Grade III: 10.5% and Grade IV: 5.3% of affected females, respectively. Increased hyperechogenicity and decreased number of fluid filled follicles were present on ultrasound in higher grades of OFD. There was no defined relationship between circulating levels of AMH and P4 and OFD. Genomic analysis indicated that 1085 genes are differentially expressed in ovarian tissue of OFD versus non-OFD animals with increased expression of 628 genes and decreased expression of 457 genes, respectively. Cluster analyses of these differentially expressed genes identified relationships with processes such as cell-cell adhesion, collagen regulation, steroid hormone regulation, and vascular support. Additional genomic analyses indicated that, of 706 microRNAs (small RNAs), 28 were differentially regulated in Non-OFD and OFD cattle with 23 increased in expression and five decreased in expression. Additionally, initial analyses of these microRNAs show relationships to various cancers. Single analysis of P4 and AMH in serum did not serve for ante-mortem diagnosis of OFD. However, variation in genetics between OFD positive and OFD negative cattle has led to multiple targets for continued bioinformatics studies.

Keywords: Ovarian follicular dysplasia, infertility, bovine

Longitudinal study of metritis risk in dairy cattle

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Metritis is a common and costly disease of dairy cattle. Cows that develop metritis are less likely to become pregnant and are culled at the end of lactation. The incidence of metritis is higher in primiparous heifers and lower in multiparous cows, and vaccination of cows against the common bacteria associated with metritis is effective in reducing incidence. Taken together, these observations suggests that cows may be developing immunity from previous exposure to the bacterial agents thus our hypothesis is that cows affected by metritis in previous lactations will have a lower risk of developing metritis. The objective was to determine if metritis occurrence in the previous lactation is an important predictor of metritis risk in future lactations.

A longitudinal epidemiologic study was performed. Data of disease occurrence and signalment of cows were obtained from the herd dairy management software of a large farm with a long-standing working relationship with our group. Complete lifetime data were available for 15,692 Holstein dairy cows. To determine the association between metritis in previous lactation and metritis risk in future lactations, only cows that survived to at least two lactations were included. The association between metritis in previous lactations and the development of metritis in future lactations were evaluated using logistic regression (PROC GLIMMIX SAS version 9.4). A total of 15,692 dairy cows for a combined 35,247 lactations were analyzed. Overall metritis incidence was 15.61%. Metritis was highest for primiparous heifers with 63.5 % of metritis cases occurring in the first lactation. For lactations 1 through 4, primiparous cows had metritis incidence of 20.63 % with second, third and fourth parity cows having 11.49 %, 11.54% and 11.45% metritis, respectively. Cows were also more likely to have metritis in the last lactation before being culled or died. Cows culled after their 1st, 2nd, 3rd, 4th, 5th, 6th or 7th lactations had a metritis incidence rate of 44.1%, 57.1 %, 73.4%, 82.12%, 85.11 %, 93.33%, 90.91%, respectively. The odds of leaving the herd is 1.63 higher given metritis in the first lactation compared to no infection. After removing 5,265 cows that did not make it to at least the second lactation, total metritis incidence was 11.49 %. Contrary to our hypothesis, cows that had metritis in the first parity and survived to the second parity were more likely to develop metritis again in her second lactation (odds ratio 1.61). Cows that had metritis in the first parity were more likely to be culled, but more interestingly, these cows were also more likely to develop metritis in the next lactation. This suggests that there is a stronger individual cow predisposition to developing the disease than potential acquired immunity from natural infection. This cow predisposition may be genetic or interaction with nutritional or immune status. Acquired immunity through vaccination can reduce the incidence of metritis though this may be a short-lived immunity. Further investigation on the duration of immunity from vaccination and predisposing factors are needed. This study also highlights the value of longitudinal studies in understanding risk factors for diseases especially when using genetic markers as many of the most severely affected cows would not remain in the herd when using cross-sectional sampling designs. In the case of diseases that can repeat over a cow's lifetime and may result in culling or death, such as metritis, a prevalence-incidence bias may occur and under-represent those with the disease. Overall, our findings reveal a longitudinal study design may be the most accurate method to categorize disease risk since cross-sectional studies provide differing results if one time-frame had been chosen compared to another.

Keywords: Metritis, dairy cows,

Effects of endogenous progesterone during ovarian follicle superstimulation on embryo quality and quantity in beef cows

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Despite modifications in techniques and protocols used for multiple ovulation embryo transfer (MOET) in the last couple decades, total quality embryos (TQE) recovered has remained relatively unchanged. The objective of this study was to evaluate the effects of endogenous progesterone during beef cow superstimulation on embryo quality and quantity. Thirty non- pregnant beef cows were sorted by breed, body condition, and age into 1 of 5 replicates. Presynchronization was staggered so each repicate began treatment on subsequent days and was accomplished using a 5 d CO-Synch + CIDR protocol. Nine days after estrus, ultrasound-guided dominant follicle removal (DFR) was performed concurrent with CIDR insertion and confirmation of a functional corpus luteum (d 0). Within each replicate, one-half of the cows were assigned a low progesterone (LP) treatment, and the other half were in the high progesterone (HP) control treatment. To remove endogenous progesterone, LP cows were administered prostaglandin F2α (PGF2α) at time of DFR. On d 1, cows began a timed, 13-d, superstimulation CIDR-based protocol with a total 320 mg FSH, (Folltropin-V, Vetoquinol), administered twice daily in decreasing amounts over four days. Cows were artificially inseminated (AI) twice (12 hours apart) on d 6 (2 days after PGF2α) using frozen-thawed semen from a single bull collection observed to have high success rates in previous superovulation research. Embryo recovery was performed on each replicate 7 days after first AI via non-surgical flush and embryos were evaluated by International Embryo Transfer Society (IETS) standards. Data were analyzed using the MIXED procedures of SAS. Results revealed a greater number of total embryos recovered from the HP than the LP cows (19.26 vs. 10.74, $P = 0.01$). Additionally, the HP cows had greater number Stage 4 embryos along with increased amount of quality Grade 3 and 4 embryos than the LP group (5.76 vs 2.20 $P = 0.002$; 1.87 vs 0.61, $P = 0.01$; 8.22 vs 2.89, $P = 0.01$, respectively). However, a higher percentage of each recovery from LP cows were Grade1 embryos (58.22 vs 37.32, $P = 0.03$). Moreover, the LP cows had a greater percentage of Stage7 and 6 TQE in a recovery (18.47 vs 1.22, $P = 0.01$; 10.37 vs 3.19, $P = 0.03$). These data indicate that with removal of endogenous progesterone during follicle superstimulation, the percentage of embryos recovered are of higher quality grade and are potentially more advanced in their development on a single recovery. However, while poorer in quality, these data also indicate endogenous progesterone presence during follicle maturation results in a greater number of total embryos recovered. While more research is warranted to determine the effects of the LP treatment on embryo recovery success, these data implicate that DFR in conjunction with removal of endogenous progesterone during superstimulation is, at minimum, a viable alternative to traditional superovulation protocols. These data also highlight the need to identify if proportional improvements in embryo quality affect transfer success.

Keywords: Corpus luteum, dominant follicle removal, embryo, superovulation

Endometrial cytokine gene expression in normal versus endometritic postpartum dairy cows

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Our objective was to better understand the inflammatory changes associated with uterine involution during the postpartum period and compare the changes that occur during normal involution versus those with endometritis. We hypothesized that distinct, time-dependent changes occur in the expression of both pro- and anti-inflammatory cytokines during uterine involution and the expression of these cytokines is altered in uterine mucosal inflammation -endometritis. Postpartum dairy cows (n=41) from a closed herd were included in our observational cohort study to assess the changes in gene expression (using qRT-PCR) in endometrial samples harvested from the uterine body using a modified cytobrush assembly. Cows were sampled first between 29-35 and then again between 49-56 days in milk (DIM). Cows were classified as normal (n=15) or endometritic (n=12) based on >18% neutrophils on cytological smears at 29-35 DIM. In addition, another set of cows with normal involution of uterus from the same herd were either sampled at 29-35 DIM (n=8) or 49-55 DIM (n=6) as procedural controls. Endometrial cytobrush samples obtained from animals from all groups were processed for qRT-PCR to quantify mRNA abundance for interleukin (IL)-1α, IL1β, IL6, IL8, IL13, IL17, IL18, colony stimulating factor (CSF)1, tumor necrosis factor alpha (TNFα), IL1 receptor antagonist (IL1Ra), IL10, transforming growth factor (TGF) β1, TGFβ2 and TGFβ3 genes relative to gene expression of β-actin (reference gene). Gene expression in uterine samples collected from the clinical cohorts was compared by Wilcoxon Rank Sum test. Expression of β-actin gene was similar (P>0.05) in samples collected from normal and endometritic cows. Gene expression data for procedural control groups did not differ from the time-matched cohorts of the Normal cows. Therefore, these data were combined with the Normal group data (with the respective time points) for further comparisons. At 29-35 DIM, uterine samples from the Endometritic cows had higher median expression of pro-inflammatory cytokines, including IL1α (17 fold, P<0.01), IL8 (16.8 fold, P<0.01), IL1β (7.6 fold, P<0.01), CSF1 (4.4 fold, P=0.03) and TNFα (2 fold, P=0.04) than uterine samples from the Normal cows. Furthermore, uterine samples from the Endometritic cows displayed greater expression of some anti-inflammatory cytokines, including IL1Ra (12.6 fold, P<0.01) and IL10 (2 fold, P=0.04), but lower expression of one anti-inflammatory cytokine - TGFβ3 (2.6 fold, P<0.01), than uterine samples from the Normal cows. By 49-55 DIM, the only difference was the higher median expression of the IL6 (9.4 fold, P=0.01) and IL17 genes (6.6 fold, P=0.04) in uterine samples from the Endometritic cows. In conclusion, the gene expression of pro- and anti-inflammatory cytokines differed between cows (at 29-35 DIM) with endometritis (≥18% neutrophils) and those involuting normally (<18% neutrophils). Differences in gene expression changed with increasing DIM.

Keywords: Cytokines, endometritis, qRT-PCR, postpartum cows.

Flotation therapy for management of calving paralysis following dystocia in a Piedmontese cow

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The most common cause of dytocia in bovine patients is feto-maternal disproportion. In these cases, damage to the sciatic and obturator nerves in the pelvic region may result in hind limb paresis or paralysis. Cases of calving paralysis can be managed conservatively with glucocorticoids, non-steroidal anti-inflammatories and tincture of time, however, prolonged recumbency in bovine patients often leads to ischemia and muscle necrosis which results in a poor prognosis for recovery.

A 9-year-old Piedmontese cow was presented to the Michigan State University Veterinary Medical Center for a dystocia of over 12 hours duration. Vaginal examination revealed the fetus was in a cranial, dorso-sacral presentation with normal posture. Assessment of fetal viability including attempts to elicit suckle and pedal withdrawal reflexes were unsuccessful. Vaginal delivery was attempted using a fetal extractor and copious amounts of water-based lubricant. The fetus became hip-locked and 90 degree rotation of the fetus resulted in successful resolution of the hip-lock and delivery of a 136 pound, non-viable fetus. However, during delivery, the cow became recumbent and was unable to rise following parturition.

Dexamethasone (60 mg IM) was administered immediately following parturition to reduce inflammation due to dystocia. The patient was moved to a deeply bedded stall and rotated side-to-side every two hours overnight. No improvement in hind limb function was noted at 18 hours after parturition and flotation therapy was initiated. The patient was placed in the AquaCow flotation tank (AquaCow Rise System, Rodding, Denmark) and was able to stand once water was added to the tank. Dexamethasone therapy was continued at a dose of 60 mg IM once daily for three days and meloxicam was administered at 1 mg/kg PO every other day.

The patient remained in the flotation tank for 48 hours at a time with 24 hours of rest in between flotation sessions spending a total of 96 hours in the flotation tank over five days. During treatment, she gradually regained function of her hind limbs and was able to stand on her own at seven days postpartum. At eight days postpartum, the patient was able to walk onto a trailer and return home. Although flotation tank management of calving paralysis cases can be time consuming and labor intensive, early intervention can decrease muscle ischemia and necrosis leading to improved case outcomes and return to productive life for bovine patients.

Keywords: Dystocia, calving paralysis, flotation therapy

Uterine microbiome, antibiotic resistance genes and virulence factors of metritic treated cows that cure or failed to cure from metritis

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Metritis is major postpartum disease in dairy cows causing reduced milk production, impaired fertility, and substantial economic losses. Antibiotics are the main therapeutic option, however, ~35% of the cows fail to recover from the disease after treatment. Furthermore, ceftiofur, the major drug used to treat a multitude of dairy cows diseases, has been linked to emergence and dissemination of antibiotic resistance for β-lactamase CMY-2. Ampicillin is an efficacious alternative to treat metritis with similar, but faster cure rates when compared with ceftiofur. However, ampicillin is also a β-lactam antibiotic that binds to specific penicillin-binding proteins belonging to the same antibiotic cluster as ceftiofur. Although from a practical standpoint ampicillin is an effective alternative to treat metritis, it is unclear if ampicillin can mitigate the selective pressure of ceftiofur, influence antimicrobial resistance genes dissemination or it is related differently to pathogens virulence factors. Herein, we used whole genome shotgun sequencing to shed light on uterine microbiome, antimicrobial resistance genes (ARGs), and virulence factors genes (VFGs) of cows that cured or failed to cure from metritis after treatment with ceftiofur or ampicillin. A cohort of 24 metritic primiparous cows healthy and not exposed to dry cow therapy and were randomly allocated to receive either ampicillin trihydrate (n = 12) or ceftiofur hydrochloride (n =12) for 5 days. Uterine swab samples for each cow were collected at metritis diagnosis (d1) and five days later (d6) one day after treatments finished. Half of the cows (12/24) recovered after treatment (ampicillin = 7 and ceftiofur = 5). Our analysis revealed that over time (from d1 to d6) the mean relative abundance (MRA) of the genera *Bacteroides, Prevotella, Alistipes, Fusobacterium,* and *Tannerella* were reduced ($P < 0.01$), whereas *Porphyromonas* was increased ($P < 0.01$) independent of treatment ($P > 0.05$). For cows that recovered from metritis, only *Streptococcus* MRA was increased when compared with counterparts that did not recover from metritis. We found the beta-diversity of microbiome communities follow a similar pattern, with microbiome diversity decreasing ($P < 0.01$) after treatment independent of treatment type ($P > 0.05$) and cure status ($P > 0.05$). Antibiotic treatment independent of type decreased VFGs abundance, but increased ARGs abundance. The resistome of metritic cows was dominated by tetracycline resistance genes, but beta-lactam ARGs such as CMY-2 were not affected by treatment or time ($P > 0.05$). The ARGs TetT and TetW increased over time ($P < 0.01$) independent of treatment ($P > 0.05$) or recovery status ($P > 0.05$). A higher MRA of VFs such as translation elongation factor Tu for *Streptococcus spp.* and heat shock protein 70 from *Vibrio cholerae* suggests that further investigation of the potential role of these bacteria and VFs on metritis pathogenesis is warranted. In conclusion, antibiotic treatment over time independent of type altered uterine microbiome, reduced VFGs abundance, and increased ARGs abundance, thus ampicillin is an alternative to ceftiofur for metritis treatment that has not direct impacts on uterine microbiome, AGRs, and VFGs.

Keywords: Metritis, microbiome, antibiotic resistance gene, virulence factors, cure.

Performance of the IDEXX Rapid Visual Pregnancy Test for pregnancy diagnosis in sheep

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Pregnancy-associated glycoproteins (PAG) belong to a family of inactive aspartic proteinases which are produced by the binucleate giant cells of the ruminant placenta. Pregnancy-associated glycoproteins are secreted by the fetal trophoblast throughout gestation and are detectable in maternal circulation allowing for pregnancy diagnosis via commercially available assays. Although the available assays are marketed for pregnancy diagnosis in cattle, similarities in PAG structure across ruminant species allow detection of PAG in other ruminants, including sheep, using the bovine assays. Previous studies evaluated the use of PAG assays for pregnancy diagnosis in sheep performed in a laboratory setting. In these studies, samples were collected, sent to a laboratory, and results were reported after several days.[1] Recently, the availability of the IDEXX Rapid Visual Pregnancy Test kit (IDEXX; Westbrook, ME) provided a method of pregnancy diagnosis with results reportable the same day as sample collection. This test could be valuable to veterinarians and sheep producers as an alternate means of rapid pregnancy diagnosis. The objective of our study was to compare results of the IDEXX Rapid Visual Pregnancy Test in sheep to pregnancy diagnosis by ultrasonography. Commercial crossbred sheep (n=143) were used for this study. Ewes had been exposed to a ram for breeding for a 60-day breeding period and rams were removed 30 days prior to ultrasound examination. Ewes were restrained in a head chute and pregnancy diagnosis performed using a variable frequency sector transducer and the Ovi-Scan sheep ultrasound (BCF Technology, Rochester, MN). Ewes were recorded as open or pregnant with an estimation of gestational age based on fetal measurements. Blood was collected into a serum separator tube via the jugular vein for analysis. Serum samples were analyzed using the IDEXX Rapid Visual Pregnancy Test kit and compared to ultrasound results. Sensitivity and specificity of the Rapid Visual Pregnancy Test were 96.8% and 73.3%, respectively, and accuracy was 94.4%. The lower specificity may be due to assay detection of early pregnancies not yet visible on ultrasound, errors in reading results of a visual assay, or the low number of open ewes in the sample group. However, the accuracy of the assay compared to ultrasound examination indicates that this test provides a reliable and cost effective method of pregnancy diagnosis in sheep which is important for both veterinarians and sheep producers seeking to improve reproductive performance in flocks.

Keywords: Pregnancy-associated glycoproteins, sheep, pregnancy diagnosis

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Evaluation of 48 hour cooled-storage after thawing frozen stallion semen

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Semen collected from stallions can be extended and used for immediate insemination, extended and stored at 5 to 8°C for 24 to 48 hours prior to insemination, or cryopreserved and stored indefinitely in liquid nitrogen. Breeding mares with cryopreserved semen requires specialized equipment for storage and thawing of semen and more intensive mare management. However, not all equine breeding programs are equipped or able to utilize frozen semen. Consequently, the objective of this study was to evaluate the longevity of frozen stallion semen once it has been thawed, extended and maintained at 5°C for up to 48 hours. Eight ejaculates from five healthy Quarter Horse stallions were utilized. Semen was collected and two aliquots were cooled in INRA96 (IMV Technologies, Maple Grove, MN) at a concentration of 50 million sperm/mL as non-cryopreserved controls. The remainder of the sample was frozen in CryoMax LE® (Animal Reproduction Systems, Chino, CA) extender at a concentration of 200 million/mL. Treatment groups were: Group 1 - straws thawed in a 37°C water bath for 30 seconds; Group 2 - straws were thawed in a 37°C water bath for 30 seconds, followed by centrifugation at 400 x *g* for 10 minutes; Group 3 - straws were thawed in a 37°C water bath for 12 seconds (time required to reach a temperature of 5°C). Semen samples were subsequently diluted to a concentration of 50 million/mL in INRA96 and cooled to 5°C. Sperm motility was evaluated at 24 and 48 hours using computer assisted sperm analysis (CASA; SpermVision®, MOFA Global, Verona, WI). Statistical analysis was performed using a one-way ANOVA, with p-value set at <0.05. Data is presented as mean ± SD. Total sperm motility for the non-frozen control semen at 24 and 48 hours of cooled-storage (77.1 ± 6.9 and 75.4 ± 9.2 %, respectively), was higher than all frozen-thawed treatment groups ($p<0.05$). There was no difference ($p>0.05$) in total motility at 24 or 48 hours of cooled-storage post-thaw between Group 1 (50.3 ± 9.6 and 51.6 ± 12.8 %), Group 2 (48.6 ± 10.1 and 47.0 ± 13.9 %), and Group 3 (32.8 ± 6.6 and 41.5 ± 6.5 %). In summary, frozen stallion sperm can be thawed, extended and cooled at 5°C for up to 48 hours and still maintain acceptable (i.e. > 30 %) total motility. Potentially, frozen semen could be thawed, extended and shipped by overnight courier to another location to inseminate into a mare. A breeding trial is clearly warranted to evaluate the fertility of frozen-thawed stallion semen after cooled-storage.

Keywords: Stallion, semen, frozen, cooled-storage

Porcine and recombinant zona pellucida vaccines as immunocontraceptives for donkeys in the Caribbean

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Immunocontraception has been investigated as an alternative to hormone manipulation and lethal methods of fertility control in wildlife for almost 30 years. Porcine zona pellucida (pZP) vaccine has been shown to utilize an animal's immune system to prevent pregnancies temporarily and reversibly in many species. This study investigated pZP vaccination as a method of practical population control in feral donkeys (*Equus asinus*) in the tropics. In addition, the study investigated a novel recombinant zona pellucida vaccine (recZP) as an alternative for fertility management. Twenty-five feral female donkeys of proven fertility were captured on Nevis and transported to Ross University School of Veterinary Medicine (RUSVM). All animal protocols were approved through RUSVM IACUC review. Jennies allocated to Group 1 (n=9) received the recZP vaccine in complete Freund's adjuvant (CFA) and thereafter two booster vaccines in incomplete Freund's adjuvant (IFA) 5-weeks and in sterile saline 10-weeks later. Jennies allocated to Group 2 (n=8) received the pZP vaccine in CFA and one booster in IFA 5 weeks later. Those allocated to Group 3 (n=8) received an initial injection of CFA and second injection of IFA 5-weeks later and acted as controls. All treatments were administered intramuscularly by injection into the left and right gluteal muscles (first and second injections), and the left pectoral muscles (third injection in Group 1). Rectal temperature, pulse, respiration, and body wall thickness of injection sites were recorded for each jenny for five days after injections. Trans-rectal ultrasonography was performed weekly on each jenny to monitor ovarian and follicular development and cyclical reproductive activity. Five weeks after the final injection, one jack was placed with each group of jennies. Estrus detection, mounting behaviors, and breeding were recorded when observed. Weekly trans-rectal observations were continued to visualize the reproductive tracts and ovaries and to identify pregnancies. Jacks were rotated through the groups every three weeks. All donkeys were cycling normally prior to injection with obvious estrus signs detected when in proximity to a jack. Following injection, all donkeys showed increased rectal temperatures and injection site body wall thickness. Sterile abscesses at injection sites were observed in 9/9 (100%) jennies in Group 1, 7/8 (87.5%) jennies in Group 2 and 3/8 (37.5%) jennies in Group 3. Four months after the final injection, 7/9 (77%) jennies in Group 1 and 6/8 (87%) jennies in Group 2 had no detectable follicular development nor estrus signs. No pregnancies were found in either of the treatment groups at sixteen weeks following treatment. In contrast, 8/8 (100%) of the control group were pregnant and all had shown follicular development. Despite the injection site reactions, both pZP and recZP vaccines were shown to be effective as a non-hormone based contraceptive with potential for application in fertility management programs in donkeys. Further investigation of these vaccines, using alternative adjuvants providing similar contraceptive efficacy without the associated adverse reactions, may benefit future population control programs for feral donkeys in the Caribbean.

Keywords: PZP, donkey, immunocontraceptive, fertility, population, Caribbean

Adenoviral vectored gonadotropin releasing hormone vaccine for temporary estrus suppression in mares

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The aim was to evaluate the immunogenicity of an adenoviral vectored anti-gonadotropin releasing hormone vaccine (Ad-GnRH) and physiological effects on cyclicity and estrous behavior in mares. We hypothesized that: 1) Ad-GnRH would induce antibodies targeting GnRH that would impede GnRH-stimulated gonadotropic activity; and 2) temporarily suppress cyclicity and estrous behavior so that mares return to cyclicity during the subsequent ovulatory season. The study took place over a 70 week period. Ten mares were assigned to treatment (n=5), and control (n=5) groups. Treatment mares were vaccinated twice, four weeks apart using Ad-GnRH. The vector was replication-deficient (Ad5, E1/E3 deleted) and was engineered to express multimerized GnRH fused with a highly antigenic carrier, leukotoxin, and enhancer elements. Cyclicity and estrous behavior were assessed twice weekly by transrectal palpation and ultrasound examination of the reproductive tract, and teasing to a stallion. Venous blood was collected weekly for progesterone and GnRH antibody assays. Following vaccination, four of five treatment mares displayed normal cyclicity and estrous behaviour that was not different from controls (Interestrus Interval (IEI) ± SEM: 23.6 ± 1.4 days). One treatment mare experienced two consecutive prolonged luteal phases that lasted 70 days and 91 days, respectively. GnRH antibodies were detected following the Ad-GnRH homologous prime-boost vaccination and maintained for 32 weeks, after which they returned to baseline. Because the GnRH antibody development to the homologous Ad-GnRH prime-boost did not alter cyclicity or estrous behavior, a heterologous boost with a single sub-effective dose (100 µg) of a protein based GnRH vaccine (Equity® TM, Pfizer Animal Health P/L, West Ryde, NSW, Australia) was administered at week 49. Two naive mares received the same dose of Equity® to control for anti-GnRH response to this antigen. By four weeks after the heterologous boost, all Ad-GnRH immunized mares became acyclic and displayed inconsistent estrous behavior that prevented calculation of IEI. All treatment mares remained acyclic for the remainder of the study period (17 weeks). Equity® control mares experienced a low GnRH antibody response that was sustained for five weeks, before returning to baseline. These control mares cycled normally, and displayed normal estrous behavior (IEI 27.3 ± 2.8 days). This study demonstrates that adult mares develop GnRH antibodies following homologous prime-boost immunization with Ad-GnRH, but antibody response was either inadequately immunogenic, or did not reach the threshold required for disruption of normal cyclicity or estrous behavior. In contrast, a heterologous prime-boost GnRH immunization strategy induced an immunogenic antibody response above the required threshold needed to suspend reproductive cyclicity.

Keywords: Gonadotropin releasing hormone, estrus suppression, mare, adenovirus

Uterine clinical findings, fertility rate, leucocyte migration and amount of COX-2 protein in endometrial tissue of susceptible mares treated with PRP at different moments of estrous cycle

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Persistent mating induced endometritis (PMIE) is an important cause of fertility decrease in horses, thereby causing a significant impact in the horse industry. A modulator of the inflammatory response that has been largely used in veterinary medicine is the platelet-rich plasma (PRP) that acts directly on inflammatory mediators. Thus, the present study aimed to investigate the effect of platelet-rich plasma on 1) uterine inflammation, conception rate, and endometrial polymorphonuclear cells (PMNs) migration, 2) the amount of COX-2 protein in the endometrial tissue, and 3) the best moment to use the PRP treatment, before or after artificial insemination (AI). A total of 13 mares classified as susceptible to PMIE were used. The mares were inseminated with fresh semen in three consecutive cycles in a cross-over study design. Platelet-rich plasma was prepared by single centrifugation protocol (120 *g*/10min). The cycles were classified as control cycle (C): no pharmacological interference; PreAI: 20 mL of PRP was infused 24 hours before AI; PostAI: 20 mL of PRP was infused four hours after AI. Artificial insemination was performed 24 hours after ovulation induction with 1mg of deslorelin acetate. Intrauterine fluid (FLU) was evaluated by ultrasonography, before and 24 hours after AI; PMNs in uterine cytology (CYT) and biopsy (HIS) were also observed before and 24 hours after AI; pregnancy diagnosis were performed 14 days after ovulation. Number of COX-2 positive cells was evaluated using immunohistochemistry by the number, intensity and the location of the labeled cells. Continuous variables were submitted to variance analyses and conception rates were evaluated by logistic regression model. Significance was set at $p \leq 0.05$ for all tests. Both PRP treatments were able to reduce ($p<0.05$) the PMNs number in CYT after breeding compared with the control cycles. Intrauterine fluid did not differ ($p<0.05$) between cycles, however the conception rate was higher ($p<0.05$) when mares were treated with PRP (63.5% - 16/25) compared with the control cycle (31% - 4/13). The number of positive mares to endometritis in the HIS, decreased ($p<0.05$) in both treated cycles and a more intense ($p<0.05$) positive COX-2 labelling was observed in the control cycle compared to the PreAI and PostAI cycles. In conclusion, there is a potential benefit of PRP to reduce the inflammatory response in PBIE mares independent of the time of treatment, increasing the chances of achieving a pregnancy in this group of mares.

Keywords: Endometritis, equine, embryo transfer, PRP.

Sexual development, productivity and reproductive abnormalities in yearling beef bulls

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Identification of young sires with advanced sexual development and improved productivity is desirable by the beef industry. However, improved productive performance seems antagonistic to sexual development and associated with reproductive abnormalities. Thus, our objectives were 1) to investigate the relationship between productive performance and sexual development and 2) to understand the relationship between mineralization in the testicular parenchyma with productive performance and fertility-related traits. Yearling crossbred beef bulls (year 1=34; year 2=43) underwent productive performance and reproductive evaluation, which included: scrotal circumference, testes echogenicity, sexual and metabolic hormones, scrotum thermography and semen quality. At slaughter, testes, vesicular glands, pampiniform plexus and epididymis were collected and processed for histomorphometry. Testicular tissue of bulls with hyperechoic regions that were casting shadows on ultrasound were histologically observed to have focal mineralization of seminiferous tubules with or without inflammation. Bulls were classified by the presence (n=14) or absence (n=14; selected at random from the full population) of testicular mineralization. Correlations between productive performance and fertility related traits, least square means comparison between bulls with or without testicular mineralization and the probability of identifying bulls with mineralization, using logistic regression, were determined using SAS software (SAS Institute Inc., Cary, NC). Productive performance was associated with measures of sexual development; lean tissue was correlated with thyroxine (r=-0.41) and epithelial height of epididymal corpus ducts (r=0.58). Carcass fat was correlated with scrotal circumference (r=0.47), percent of sperm tail defects (r=-0.34) and vesicular gland cell area (r=-0.34). Bulls with testicular mineralization had greater ribeye area (mean ± SEM; 88.6 ± 1.8 vs. 84.6 ± 1.8 cm^2; p=0.03) and tended to have greater initial body weight (308 ± 7.6 vs. 288 ± 7.6 kg; p=0.06), and lower testicular weight (349±10.7 vs. 378±10.7 g; p=0.06). There was no difference in scrotal circumference (35.0 ± 0.5 vs. 36.1 ± 0.5 cm), sperm motility (69.2±4.1 vs. 75.2±3.8 %), normal sperm morphology (76.3±3.1 vs. 72.6±2.9 %) and surface temperature of the scrotum (30.3C ± 0.3 vs. 29.7C ± 0.3) between bulls with and without presence of testicular mineralization (p>0.10). It was determined that productivity, testicular echogenicity, scrotum thermographs and semen quality ensured a certainty of detecting a bull with testicular mineralization by more than 80%. Further analysis of testicular ultrasound, sexual and metabolic hormones and reproductive tissue histomorphometry are in progress. These preliminary results support that improved productivity relates to sexual development and occurrence of abnormalities in the reproductive system of young sires.

Keywords: Scrotum thermographs, semen quality, sexual and metabolic hormones, testicular mineralization, testis echogenicity

Next Generation DNA Sequencing, culture and cytology results in 10 clinically normal mares

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Endometritis, a common cause of infertility in the mare, is traditionally diagnosed with aerobic culture and cytologic evaluation of fluid, swabs, or tissue obtained from the uterine lumen or endometrium. Next Generation DNA sequencing (NGS) is a method of microbial diagnostics using 16S sequencing that is used in human medicine to identify microorganisms not easily identified by traditional culture techniques. The objective of this study was to describe the Next Generation Sequencing (PathoGenius Laboratory, Lubbock, TX) results compared to traditional methods of diagnosing infectious endometritis in a group of clinically normal mares. A group of 10 clinically normal recipient mares (weight 500-700 kg, mean age 7.3 years, 2 barren, 1 maiden, and 8 mares had foaled > 4 months, and been weaned >30 days prior to sampling) were used as subjects. Transrectal palpation and ultrasonography was performed and samples obtained from subjects with: one ≥ 30mm follicle present on an ovary, moderate uterine edema, <1 cm anechoic fluid in the dorso-ventral plane, and a moderately relaxed cervix. Endometrial swabs (ES) were obtained through a vaginal speculum using a double-guarded Kalajian swab, one swab was placed in Amies medium and submitted for aerobic culture, the other swab in a red top tube was submitted for NGS. Endometrial cytology using the cap from a double-guarded swab was obtained and submitted for cytologic analysis as previously described.[1] Small-volume lavage (SVL) of the uterine lumen was performed using 1 L of sterile lactated Ringer's solution, and the efflux was split into two 50 mL conical tubes, 1 submitted for aerobic culture and cytology, the other submitted for NGS. An endometrial biopsy was obtained and swabbed and submitted for aerobic culture and NGS. Quantitative PCR was performed prior to NGS to indicate microbial load, NGS sequencing was reported as: negative = no microbial DNA isolated, low = less than 10^5 bacteria/fungi/mL, or greater than 10^5 bacteria/fungi/mL and the microbial DNA isolated listed by genus, species, and the percentage of microbial DNA present. Aerobic culture of ES recovered no growth on 90% (9/10) samples, and 77.8% (7/9) of these were negative with NGS and 22.2% (2/9) were low, (*E.ictaluri* and *S.epidermis* were the primary microbes identified). Aerobic culture of 10% (1/10) of ES recovered a *Bacillus* species with a low NGS result (*Corynebacterium* the majority of DNA identified). Aerobic culture of SVL recovered no growth on 70% (7/10) of samples, and 30% recovered growth of one organism (*E.coli*, *α-streptococcus*, and *β-streptococcus*). Next Generation DNA sequencing analysis of SVL recovered negative results on 70% (7/10), and low results on 30% (3/10) with *E.coli, P.aueruginosa, C.xylanorovans* isolated, none of which grew on aerobic culture. Swab of endometrial biopsies recovered no growth on aerobic culture of all samples, and only one sample NGS detected low levels of *B.simplex.* Cytology results from endometrial swabs were 80% (8/10) normal, and 20% (2/10) had moderate inflammation. Cytology results from SVL recovered no neutrophils on 60% (6/10) of samples and 1-2 leukocytes/100x magnification on 40% (4/10) of samples. The present preliminary study describes the NGS results compared to traditional methods for confirming bacterial endometritis in a normal population of mares. Further controlled studies are required to determine the effectiveness and utility of this diagnostic tool in identifying clinical and subclinical cases of infectious endometritis.

Keywords: Endometritis, equine, Next Generation Sequencing

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Effect of repeated small follicle oocyte collections on the follicle population and gross ovarian changes in mares

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Equine oocyte collections using transvaginal aspirations (TVA) can be used in clinical settings to obtain oocytes for oocyte transfer or intracytoplasmic sperm injection (ICSI). Oocytes are often collected from all ovarian follicles >5mm in diameter on both ovaries (SFTVA). In parallel to the increased demand for this procedure, concerns regarding the effects on long-term fertility and overall safety of donor mares have been raised. We hypothesized that repeated aspiration of all follicles from mare's ovaries could induce ovarian trauma and fibrosis and, potentially, limit the reproductive lifespan of young fertile mares. Our first aim was to determine if gross ovarian changes are associated with repeated SFTVA. Our second aim was to determine the repeatability of success of SFTVA from a single ovary. Mares (n=6), 5-12 yr., were assigned to the study in 2015. Follicular activity was monitored by transrectal ultrasonography. Aspirations of all small follicles >5mm were performed on the left ovary only of all mares, no manipulation occurred to the right ovary. Following the conclusion of the 2015 breeding season, four of the mares were subjected to a standing laparoscopic bilateral ovariectomy. The remaining two mares continued with the same protocol throughout the 2016 season (April to September) prior to ovariectomy. Follicle numbers and diameters were compared between aspirated and nonaspirated ovaries using a paired t-test. The mean number of follicles imaged by ultrasonography per ovary and month, was not statistically different in 2015 (P=0.58) or 2016 (P=0.51). The mean follicle diameters per month, based on ultrasound imaging, were different (P=0.04) between the left and right ovaries for the six mares in 2015 (P=0.04), but not the two mares in 2016 (P=0.24). The mean number of aspirations per mare was 9.38 with an interval of 19.1 days between procedures for both years. The mean number of follicles punctured per aspiration session was 4.8 (±0.28), resulting in an average of 1.9 (±0.2) oocytes, yielding a 40% recovery rate per session. Prior to removal, a laparoscopic examination of ovarian ligaments and oviducts was performed. Once removed, the gross size of the aspirated (left) ovary was notably smaller than the control (right) ovary in two out of the four mares that underwent surgery in 2015. Both mares had grossly smaller left ovaries after removal in 2016. For all six mares, the aspirated ovaries were palpably firmer with rectal palpation and digital palpation after surgical removal. Histological data are pending; however, the use of repeated small follicle aspirations did appear to affect the texture, follicle diameter and overall ovarian size compared to nonaspirated ovaries. By utilizing only one ovary for SFTVA, a mare's long-term reproductive health could be preserved by sparing the other ovary for normal functionality.

Keywords: Mare, follicle, aspiration, ovary, oocyte

Effect of human chorionic gonadotropin (hCG) treatment on the duration of oxytocin-induced prolonged corpus luteum (CL) function in mares

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Two protocols are now available for using oxytocin treatment to induce prolonged CL function to suppress estrous behavior in performance mares.[1] One protocol involves determining the day of ovulation, and administering 60 units of oxytocin intramuscularly (IM) once daily on days 7 to 14 after ovulation. The other protocol does not require determining the day of ovulation, but requires administration of 60 units of oxytocin IM once daily for 29 days when treatment is started randomly during the estrous cycle. Both protocols are equally effective and induce prolonged CL function in 60 to 70% of treated mares. When mares develop prolonged CL function, the CL remains functional for 60 to 90 days. However, when using oxytocin treatment to induce prolonged CL function for estrus suppression, a longer duration of CL function would be beneficial. We hypothesized that administration of hCG during the period of oxytocin-induced prolonged CL function would extend the duration of CL function through two mechanisms: 1) a direct luteotrophic effect and/or 2) by inducing ovulation of a diestrus follicle(s) resulting in the formation of a new CL(s) that would function for an additional 60 to 90 days. Therefore, the objective of this study was to determine if administration of hCG during the period of oxytocin-induced prolonged CL function would extend the duration of CL function. Prolonged CL function was induced in 14 mares by administering 60 units of oxytocin IM once daily on days 7 to 14. Mares were randomly assigned equally to a control group that received no additional treatment and an hCG-treated group that received 2,500 units of hCG IM on days 30, 45, 60, 75 and 90 after ovulation. Jugular blood samples were collected on the day of ovulation and then three times weekly (M, W, F) for 120 days for determination of blood progesterone concentration. The duration of CL function (progesterone >1.0 ng/mL) was compared between control and hCG-treated mares using the Wilcoxon Rank Sum test. The duration of CL function was 78.0 ± 2.8 and 91.4 ± 20.4 days (mean ± SD) in control and hCG-treated mares, respectively, which was not significantly different ($P>0.05$). Therefore, this study found no benefit of administering hCG during the period of oxytocin-induced prolonged CL function, so alternative methods of extending the duration of CL function should be explored.

Keywords: Equine, mare, oxytocin, corpus luteum, hCG

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Enrofloxacin and its active metabolite (ciprofloxacin) cross the equine placenta and do not cause lesions in the fetal articular surface and growth plates

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Antimicrobial therapy is used in broodmare practice to treat a variety of bacterial diseases including pneumonia, septic arthritis, osteomyelitis, enterocolitis, and placentitis. Unfortunately, evolving microbial resistance to commonly used antimicrobials has decreased the number of affordable and reliable antibiotics available to treat these infections. Enrofloxacin, a fluoroquinolone antimicrobial with bactericidal activity against both gram–negative and gram–positive pathogens, would be an excellent therapeutic option for bacterial infections in pregnant mares if proven safe. There is currently no evidence that enrofloxacin crosses the equine placenta and therapeutic use is largely avoided in pregnant mares as it is assumed that enrofloxacin is toxic to the fetus based on limited *in vitro* and foal studies. We hypothesized that enrofloxacin and its active metabolite (ciprofloxacin) cross the equine placenta without production of cartilaginous lesions in fetuses. The objectives of this study were to determine enrofloxacin and ciprofloxacin concentrations in the maternal and fetal plasma and fetal fluids when enrofloxacin is administered at therapeutic doses during late-term pregnancy, and to evaluate the articular cartilage of the long bones of fetuses from those mares. Mares carrying normal pregnancies (~280d of gestation) were assigned to: 1) control (n=3), 2) therapeutic dose of enrofloxacin (n= 6, 5mg/kg), and 3) double therapeutic dose enrofloxacin (n=6, 10mg/kg). Enrofloxacin was administrated intravenously every 24h for 10d. Maternal plasma samples were collected daily for 11d and plasma preserved at -80°C. Transabdominal ultrasound guided fetal fluid sampling was performed at days 1, 5 and 11. Premature delivery was induced by manual dilation of the cervix and serial doses of oxytocin. At delivery, fetal plasma was collected and similarly preserved. Enrofloxacin and ciprofloxacin were measured by LS-MS/MS. Proximal articular surfaces of humerus, radius, femur, and tibia were examined macroscopically and histologically. Statistical analysis was performed using ANOVA with repeated measures and significance was considered when $p<0.05$. Enrofloxacin and ciprofloxacin reached minimal inhibitory concentrations for common equine pathogens in all examined fluids. Ciprofloxacin did not significantly increase with the double pharmacological enrofloxacin dose in maternal plasma, but allantoic fluid showed a 10-20 fold increase relative to maternal and fetal plasma concentrations. No macroscopic, cytological, or extracellular matrix lesions were found in the fetal cartilage from enrofloxacin-treated mares. These findings support our hypothesis that enrofloxacin and its metabolite cross the equine placenta and do not induce cartilaginous lesions in the fetus at recommended doses in late pregnant mares. Additional studies are warranted to confirm our findings of lack of fetal injury.

Keywords: Fetal toxicity, late-term pregnancy, fluoroquinolone

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Serum and hair testosterone concentrations do not differ in stallions between social ranks

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In many species, male social rank has been found to correlate with testosterone concentration ([T2]), with more dominant males having higher circulating [T2]. However, the influence of [T2] on social ranking has not been evaluated yet in feral horse herds. The purpose of this study was to compare [T2] and cortisol concentration ([CORT]) in serum and hair in stallions from a variety of different social ranks. We hypothesized that dominant stallions would have higher [T2] and submissive stallions would have higher [CORT]. We also hypothesized that there would be a correlation between serum and hair [T2] and [CORT]. Stallions used in this study were part of the feral horse herd managed on the Confederated Tribes of Warm Springs Reservation (n=15). After gathering stallions for the purpose of castration, behavioral evaluations were conducted on each horse in a small and large pen to determine how they interact with other stallions. A 5-point behavior score was created with 1 = highly submissive and 5 = highly dominant. Samples were collected when each stallion was anesthetized. A jugular venous blood sample was collected, allowed to clot, and serum was stored at -20°C. A mane hair sample was pulled out by the root. [T2] and [CORT] were extracted from the hair as previously described.[1] Briefly, 100 ± 20 mg of hair was weighed, minced into 3-4 mm pieces, sonicated in methanol (2 mL) at 20°C for 30 minutes, and incubated overnight at 50°C in a water bath with gentle shaking. The methanol was pipetted off into a new glass vial and evaporated to dryness under nitrogen. The samples were then reconstituted with 125 µL of assay buffer (#80-0170, Assay Designs, Inc., Ann Arbor, MI). Chemiluminescence (Immulite 1000, Siemens Healthcare Diagnostics, Tarrytown, NY) was used to measure [T2] and [CORT] from serum and extracted hair samples. The behavior score collected from both pens was averaged and then categorized as either submissive (<2.5), neutral (2.5-3.5), dominant (>3.5). The behavior score category was compared using an ANOVA for serum and hair [T2] and [CORT]. In addition, a Pearson correlation analyses was performed to determine if there was an association between serum and hair [T2] or [CORT]. Significance was defined as $p<0.05$. There were no significant differences between hair (ng/100mg) and serum (ng/mL) [T2] or [CORT] in submissive, neutral, or dominant stallions (mean ± standard deviation reported in the table below). There was also no correlation between serum and hair [T2] or [CORT] (R^2= 0.0257, and 0.0025, respectively).

Behavior	Serum [T2]	Hair [T2]	Serum [CORT]	Hair [CORT]
Submissive	35.3 ± 26.5	188.6 ± 89.6	10.2 ± 5.4	1.0 ± 0.0
Neutral	35.9 ± 26.0	173.4 ± 43.0	9.4 ± 3.0	1.0 ± 0.0
Dominant	71.0 ± 54.2	183.5 ± 42.6	10.9 ± 1.4	1.3 ± 0.6

While it was unexpected that [T2] and [CORT] did not differ between social ranks, this may reflect co-dependencies that exist within bachelor bands and warrant further research.

Keywords Behavior, chemiluminescence, cortisol, dominant, submissive.

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Validation of a fixable stain for assessing viability of stallion sperm

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Most sperm viability assays utilize unfixed sperm. Fixable stains have been developed to evaluate the viability of somatic cells but this methodology has not been validated for equine sperm; hence, the objective was to compare a fixable stain with conventional methods. We hypothesized that sperm viability measures are similar when comparing staining techniques for fixed and unfixed equine sperm. Using a protocol adapted in our laboratory, a fixable staining method was compared with commonly used methods for examining sperm viability in unfixed specimens, i.e, 1) SYBR-14 and propidium iodide (SYPI), 2) *Pisum sativum* agglutinin and PI (PSAPI), 3) NucleoCounter (NC) which utilizes PI, and 4) eosin nigrosin (EN). Ejaculates (n=34) were obtained from 10 stallions. Initially, sperm concentration and percent viable sperm were determined using an automated cell counter (NucleoCounter®SP-100™, ChemoMetec A/S, Allerød, Denmark). Aliquots of fresh semen were diluted to 30 x10^6 sperm/mL in INRA96 extender (INRA 96; IMV, Maple Grove, MN). Semen treatments for analysis were based on different contributions of fresh sperm and flash-frozen sperm (from a single source and diluted to 30 x10^6 sperm/mL in INRA96 extender). Treatments included: 1) 100% fresh sperm (T100); 2) 50% fresh and 50% frozen-thawed sperm (T50); and 3) 0% fresh sperm (T0). For T100, samples were analyzed using the LIVE/DEAD® Fixable Red Dead Cell Stain Kit (Thermo Fisher Scientific, Waltham, MA; FLD), and PSAPI, SYPI, NC, and EN assays. For T50 and T0, samples were analyzed using the FLD, PSAPI, and SYPI assays only. Samples processed for the FLD, PSAPI, and SYPI assays were evaluated by flow cytometry. For the EN assay, semen and stain were mixed on a microscope slide and 100 sperm were scored using microscopy. The relationships between FLD and all unfixed viability assays were determined using correlation statistics (PROC CORR, SAS Institute, Cary, NC) and Bland-Altman analysis. For T100, correlations (r) between FLD and NC, PSAPI, SYPI, and EN assays were 0.90, 0.92, 0.89, and 0.87, respectively ($P<0.05$). The mean, standard deviation and range of the difference for FLD-NC were 0.4, 6.6, -17 to 13%; for FLD-PSAPI were -2.4, 5.7, -19 to 8%; for FLD-SYPI were -2.3, 7.4, -21 to 14%; and for FLD-EN were -9.4, 7.5, -26 to 2%, respectively. For T50, correlations (r) between FLD and PSAPI or SYPI were 0.82 and 0.77, respectively ($P<0.05$). The mean, standard deviation and range of the difference for FLD-PSAPI were -5.6, 4.5, -25 to 1% and for FLD-SYPI were -5.3, 5.4, -27 to 7%, respectively. For T0, correlations (r) between FLD and PSAPI or SYPI were 0.93 and 0.89, respectively ($P<0.05$). The mean, standard deviation and range of the difference for FLD-PSAPI were -2.0, 0.5, -2.8 to -0.9% and for FLD-SYPI were 0.2, 0.2, -0.2 to 0.7%, respectively. This study describes a viability assay in which equine sperm can be fixed immediately following semen collection. Correlations between fixed and unfixed flow cytometric assays were high and absolute values were generally similar. The EN stain tended to overestimate viability, as compared to the FLD assay. The FLD assay allows for assessment of initial sperm viability whereby practitioners can fix semen samples immediately following ejaculation and send to a reference laboratory for analysis.

Keywords: Equine, sperm, viability, assays, fixation

Comparison of maturation, cleavage and blastocyst rates in equine oocytes recovered by transvaginal aspiration from estrogen-subordinate follicles versus non-estrogen-subordinate follicles

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The *in vitro* production of equine embryos provides an alternative method for the preservation of valuable genetic lines. To optimize efficiency, oocytes are often collected from donor mares by transvaginal aspiration (TVA) every two weeks during which time the donor mares do not undergo estrogenic stimulation of the estrous cycle. However, the hormonal stage of the mare at the time of TVA of oocytes has been shown to affect the chromosomal and cytoplasmic characteristics of oocytes. Juvenile chromatin and higher mitochondrial activity appear to be more often associated with immature oocytes recovered from new wave follicles as opposed to oocytes from follicles that are under estrogenic stimulation (estrogen-subordinate).[1] The purpose of this study was to determine if there was a difference in maturation, cleavage and blastocyst development rates between oocytes recovered from estrogen-subordinate follicles versus follicles that are not under estrogenic and human chorionic gonadotropin (hCG) stimulation. Our hypothesis was that there is a significant difference based on endocrine stage of the mare cycle. Immature cumulus-oocyte-complexes (COC's) that were recovered from ovarian follicles <25mm by TVA every two weeks from mares not exhibiting ultrasonographic or behavioral signs of estrus (Group 1; n =301)) and from mares exhibiting signs of estrus 20-22 hours after ovulation induction (Group 2; n =100)) were held overnight at 20^{0}C in embryo holding medium. After 28.5 hours, mature MII oocytes were denuded following exposure to 80iu hyaluronidase and fertilized by ICSI with thawed spermatozoa from a single fertile stallion. Fertilized oocytes were cultured as described by Foss et al.[2] Data were analyzed using the Chi-square test. Although the maturation rate, cleavage rate, blastocyst rate per cleaved and mature oocyte were higher in Group 2 compared with Group 1 oocytes ($p = 0.12$, $p = 0.22$, $p = 0.27$ and $p = 0.08$ respectively), only the blastocyst production per oocyte recovered rate was significantly increased ($p = 0.03$) in the Group 2 oocytes. The results of this study suggest that oocytes recovered from estrogen-subordinate follicles are associated with a higher blastocyst production rate when compared with oocytes recovered during nonestrus and thus, may influence optimal timing of TVA of oocytes.

Keywords: Equine; oocyte; intracytoplasmic sperm injection; blastocyst.

Acknowledgement

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Duration of gestation in Thoroughbred mares kept under tropical and subtropical climate

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In horses, luminosity and nutrition are well characterized to control reproductive cyclicity and duration of gestation. However, limited information exists on the effects of temperature, precipitation, and humidity. Therefore, we hypothesized that temperature and humidity combined affect the duration of gestation in mares kept under tropical and subtropical climate. The objective of this study was to assess the effects of temperature and humidity on the duration of gestation of Thoroughbred mares. The gestational length of 376 Thoroughbred mares carrying and delivering normal singleton foals (n = 720) were recorded and analyzed for four foaling seasons of the Southern Hemisphere. Additional data recorded included foal gender, mare age (young 3-7 years vs. mature ≥8 years) and parity (primiparous vs. multiparous). The mares were housed in three stud farms as follows: stud farm 1, (removed to preserve confidentiality, subtropical), altitude 212 m (n=400 pregnancies of 203 mares); stud farm 2, (removed to preserve confidentiality, subtropical), altitude 875m (n= 98 pregnancies of 49 mares); and stud farm 3, (removed to preserve confidentiality, tropical), altitude 626m (n= 202 pregnancies of 124 mares). Stud farm 2 was located in an intermediate geographic region between stud farms 1 and 3. All mares were submitted under similar management and nutritional conditions, i.e., kept on pasture throughout the day, supplemented with grain and kept in a stall during the night upon imminent foaling. Climatological endpoints assessed included dry bulb temperature (T_{db}), relative humidity (RH), precipitation, and the calculated temperature-humidity index (THI). The meteorological data were gathered from official national weather stations in the three different stud farms. All mares naturally covered, the ovulation was confirmed by daily per rectum palpation and ultrasonography examination. Continuous data were analyzed by ANOVA and post hoc comparisons with Tukey's test. It was considered the interactions of the location and foal gender, parity and age of the mares. Significance was set at $p<0.05$ and results expressed as mean ± SD and ranges. As previously reported colts (343 ± 10, 320-390 d, n=361 foals) had longer gestation than fillies (340±12, 321-375 d, n=359 foals) (p=0.03), with no significant effects of location, or interactions with weather conditions. As anticipated mature mares (344 ± 11, 321-390, n=342 pregnancies) had longer gestation lengths than young mares (341±11, 320-375 d, n=378 pregnancies) (p=0.009). Stud farm 1, had the longest gestation length (344±12, 320-390d) followed by stud farms 2 (338 ± 7, 321-358 d), and 3 (337±7, 320-366 d) ($p<0.001$). The RH and precipitation were similar ($p>0.05$) for stud farms 1 (73 ± 7%; 140 ± 99 mm^3, respectively) and 2 (80 ± 5 %, 140 ± 91 mm^3, respectively), but significantly higher ($p<0.001$) than stud farm 3 (67 ± 3%; 40 ± 46 mm^3, respectively) for both endpoints. The T_{db} and THI were significantly different ($p<0.001$) for all three locations (Stud farms 1 (15.4 ± 2.9°C, 60 ± 4.5) 2 (17.3 ± 6 °C, 64.4 ± 9.6), 3 (20 ± 2, 66.3 °C ±3). There were no significant interactions ($p>0.05$) between location, foal gender, parity and age of the mare. Since mares were managed similarly, we suggest that the effects observed in gestation length are likely due to temperature and consequently THI, rather than location. Humidity and precipitation appeared to not have affected gestation length.

Keywords: Pregnancy, temperature, relative humidity, temperature-humidity index

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Randomized comparative trial of electro-acupuncture and exercise versus uterine ecbolics in the treatment of persistent post-breeding endometritis in mares

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Exercise and acupuncture are frequent therapies used for the treatment of persistent post-breeding endometritis, however their actual efficacy to date is unproven. The objective of this study was to determine if exercise and acupuncture are effective methods to reduce intrauterine fluid and compare the effectiveness of these treatments to well-established uterine ecbolics including oxytocin and cloprostenol. It was hypothesized that mares receiving exercise, acupuncture, oxytocin, and cloprostenol will show increased fluid clearance compared to stall-rest control mares. Twelve mares susceptible to post-breeding endometritis were enrolled into the study and followed through six estrous cycles with a randomized crossover design utilizing both positive and negative controls. During each estrous cycle, mares were randomized into one of six treatment groups, including stall rest (SR; negative control), oxytocin (O; positive control), cloprostenol (C; positive control), exercise (E), electro-acupuncture (EA), and oxytocin and exercise (OE). Each mare was challenged with an insemination dose of 500 x 10^6 dead sperm at time 0 h. Intrauterine fluid measurements were taken at 4, 24, 48, 72, and 96 h after breeding and considered cleared if intrauterine fluid measured <2 cm in average cross-sectional depth. Associations between treatment efficacy and fluid clearance were investigated using a random-effects logistic regression model that controlled for positive uterine culture. Compared to the stall-rest negative control, exercise was the most effective treatment and had 31.6 times increased odds of fluid clearance (OR, 31.6; 95% CI, 1.9-513.0). The second most effective treatment was the combined oxytocin and exercise group that had 13.1 times increased odds of fluid clearance (OR, 13.1; CI, 1.2-143.3). This was followed by cloprostenol that had 10.6 greater odds of fluid clearance (OR, 10.6; CI, 1.1-99.6) and finally oxytocin that had 4.9 times greater odds of fluid clearance (OR, 4.9; CI, 0.6 – 40.9). Estimates of electro-acupuncture were not calculable as only five mares tolerated the treatment. To our knowledge, the present study is the first conducted that tests the efficacy of electro-acupuncture, exercise, as well as combined exercise with oxytocin to clear intrauterine fluid accumulation following breeding. Anecdotal reports have suggested that these treatment options are efficacious yet they have long remained unproven. Results from this study can confirm that exercise and exercise combined with oxytocin are effective. Interestingly, these two treatment groups were the most effective in this study though these findings might be the consequence of the small sample size.

Keywords: Equine endometritis, cloprostenol, oxytocin, exercise, electro-acupuncture

Metagenomic analysis of the equine placental microbiome

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The placenta is a vital, but transient, organ of pregnancy that has been regarded as sterile in both women and mares. Recent evidence in women has demonstrated that the placenta harbors a unique microbiome. The objective of this study was to determine if the equine placenta harbors a microbiome under healthy conditions, and if so, is there any association with the microbiomes of extraplacental body sites (oral, vaginal, or fecal) of the mares. We hypothesize that the equine placental microbiome has a distinctive metagenomic profile, not yet characterized. Fecal, oral, and vaginal samples were taken from pregnant mares within 30 days of foaling, as well as the gravid and non-gravid regions of the chorioallantois at the time of foaling (n=4). Genomic DNA was isolated from all samples, and the bacterial 16s ribosomal RNA gene was amplified by PCR. Blank extractions were amplified as negative controls, while known tissue samples were amplified as positive controls. Amplified bacterial DNA was sequenced using the Mi-Seq sequencer. Sequence reads were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) program and assigned to Operational Taxonomic Units (OTUs). These OTUs identify the genera of bacteria present in the sample. Discriminant analysis (JMP Pro 12) was used to evaluate the correlation between bacterial taxa and the prevalence of each type of bacteria in the placental samples with the potential source of bacteria from extra-placental body sites. Different prevalence of bacteria in each sample was used as a covariate in a stepwise discriminant analysis model. Variables were removed in a stepwise manner until the only variable with a $p< 0.005$ were retained in the final model. Relative abundance within the chorioallantois demonstrated 3 main phyla represented in the gravid horn (Firmicutes, Proteobacteria, Bacteroidetes) and those same three phyla plus Actinobacteria in the non-gravid horn. The most abundant phyla within the oral, fecal, and vaginal samples (Firmicutes and Proteobacteria) were also detected in the chorioallantois. The gravid horn and feces of the mare have different populations of bacteria. *Bacillus, Mycoplasma,* and *Gemella* are all higher in relative abundance in the fecal samples of the mare ($p<0.0001$). Conversely, *Clostridium* and *Moraxella* are higher in the gravid horn samples ($p<0.0001$). The most abundant bacterial phyla in gravid and nongravid chorioallantois share significant overlap, suggesting similar, but not identical, environments within different compartments of the chorioallantois. Phyla of relatively high abundance in oral and vaginal samples correspond to those found in the chorioallantois, indicating possible associations between placental and extra-placental microbiota, yet there are significant differences between the gravid horn and the fecal samples. To the authors' knowledge, this is the first report to characterize the equine placental microbiome by metagenomics. Further studies include pyrosequencing of equine placentae from mares with adverse pregnancy outcomes, i.e. placentitis and fetal growth restriction, to determine if the equine placenta has a unique microbiome in health and disease.

Keywords: Placenta, microbiome, metagenomics, pregnancy

Metagenomic sequencing of the uterine microbial environment during estrus and early pregnancy in mares

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Bacterial endometritis is the leading cause of infertility and is a condition of great concern to breeders and veterinarians. The traditional approach to diagnosis and treatment of endometritis is via bacterial culture of the uterus. With the recent developments in metagenomic approaches to study microbial populations, it had been demonstrated that in other species many 'sterile' anatomic sites harbor complex microbial populations of bacterial organisms that cannot be grown in the laboratory. We hypothesized that the healthy equine uterus is not a sterile environment but instead is colonized by a population of complex bacterial microflora. To test our hypothesis we subjected uterine fluid obtained from mares to metagenomic DNA sequencing of the 16S rRNA gene around the time of ovulation/ artificial insemination and during early pregnancy. Our study comprised of mares divided into two groups. Group A (n= 10) consisted of mares admitted for routine breeding management whereas Group B (n= 10) comprised of mares admitted for embryo flushing. Uterine flush samples were obtained from mares in Group A pre-ovulation, 12 hours post-ovulation and 24 hours post-ovulation. The pre-ovulation samples were also subjected to uterine cultures and based on results, the mares were further designated as being culture positive or negative. All uterine flushes were subjected to further metagenomic DNA sequencing. Mares in Group B were flushed at day 7 after ovulation and the uterine flushes were collected and sent for bacterial culture analysis as well as subjected to metagenomic sequencing. Data analysis was performed using the Qiime Software. Metagenomic sequencing identified over 200 bacterial species in both culture negative and culture positive samples (from Group A) demonstrating that the uterus is not a sterile site at any point during and after estrus. *Proteobacteria and Bacteroidetes* species were statistically associated with culture positive samples according to the Bonferroni correction. All mares in Group B tested negative on bacterial cultures. LEfSe comparison revealed that *Sphingobacteriales* (*Bacteroidetes*) and *Sphingobium* (*Proteobacteria*) were statistically associated with mares carrying embryos. *Rhodocyclaceae* and *Enterobacteriaceae* (Proteobacteria) were statistically associated with mares not carrying embryos. Through this pilot study we have managed to produce enough evidence of the presence of a complex bacterial microbiome of organisms that fails to grow using routine uterine culture methods. Further studies investigating the role of this background flora in maintaining uterine health, resisting pathogens or even playing a major role in pregnancy maintenance need to be pursued. Also, due to the extremely sensitive nature of this diagnostic modality, ideal sampling techniques need to be optimized to increase the specificity of data collected.

Keywords: Mare, uterine, metagenomics, sequencing, microbiome.

Estradiol cypionate aided treatment for experimentally induced ascending placentitis in mares

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The overall goal of this study was to assess the efficacy of various therapeutic combinations of a long-acting estrogen (estradiol cypionate; ECP) and a long-acting progestin (altrenogest; ALT) in addition to basic treatment for placentitis with trimethoprim-sulfamethoxazole and flunixin meglumine (TMS+FM). Specific aims for this experiment were to evaluate (i) time from induction of bacterial placentitis to delivery, gestational length, and foal parameters (high-risk, survival, and birth weight); and (ii) serum steroid concentrations (progesterone, 17α-hydroxyprogesterone, 17β-estradiol, and cortisol) in response to treatment. Pregnant mares (300 d gestation, n=46) were randomly split into healthy mares (control group, CONT, n=8) and mares with experimentally induced ascending placentitis (n=38). Placentitis was induced via intracervical inoculation of *Streptococcus equi* subspecies *zooepidemicus*. Thereafter, induced mares were randomly assigned into: (1) TMS+FM (n=8); (2) TMS+FM+ALT (n=8); (3) TMS+FM+ALT+ECP (n=6); (4) TMS+FM+ECP (n=6); and (5) no treatment (INOC, n=10). Treatments were started 48 h after bacterial inoculation and carried out for 10 d. All mares had blood samples collected and were assessed for signs of placentitis daily until foaling, or for 10 d. Steroids were analyzed via RIA. Continuous data were analyzed by ANOVA, and categorical data analyzed by Fisher's exact test. Significance was set at $p<0.05$. Mares in the TMS+FM+ECP (346 ± 5; 46 ± 4 d) and CONT (335 ± 5; 35 ± 5 d) groups had the longest gestation lengths and induction to delivery intervals. However, gestation length for these groups was similar to TMS+FM+ALT+ECP (330 ± 11; 22 ± 6 d). Foal survival at parturition and 7 d after delivery were similar across treated groups (66.7-100%), and CONT group. Similar to CONT, TMS+FM+ECP had no high-risk foals; other treated groups had higher incidences (50-75%) ($p<0.05$). The inclusion of ECP in the treatments resulted in foals with body weight similar to CONT group ($p>0.05$). There were no group effects or time by group interactions on concentrations of steroids assessed herein ($p>0.05$). In conclusion, mares with experimentally induced ascending placentitis benefited from estrogen supplementation, but progestin supplementation did not appear to make a difference in outcomes.

Keywords: Pregnancy loss, foal survival, placental pathology, estrogen, progestins

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Effect of flunixin meglumine, meloxicam and firocoxib on equine embryonic vesicle mobility

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Embryonic vesicle (EV) mobility arises as a result of prostaglandins produced by the conceptus and endometrium that promote local contraction of the uterine smooth muscle, displacing the EV throughout the entire lumen of the uterus. Administration of anti-inflammatory drugs at the time of embryo transfer is routinely used to reduce subclinical uterine inflammation and prostaglandin F2α production by the endometrium. Stout and Allen (2001) observed that EV mobility was markedly reduced immediately after an i.v. injection of flunixin meglumine (FM). Inadequate migration of the EV through the uterine lumen can impair maternal recognition of pregnancy. The aim of this study was to evaluate the effect of three nonsteroidal anti-inflammatory drugs (NSAIDs) on EV mobility: FM, an antiprostaglandin non-specific for cyclooxygenase (COX), firocoxib (FIRO), selective for COX-2 and meloxicam (ML), a COX-2 preferential. Day 12 pregnant mares were divided into three treatment groups of 10 mares/group: FM (1.1 mg /kg, IV), FIRO (0.2 mg /kg, PO) and ML (0.6 mg/kg, IV). Embryonic vesicle mobility was monitored with serial transrectal ultrasound examinations, every 5 minutes for 1 hour. The first examination was the pre-treatment control and the second examination was done after the administration and peak plasma level established of each NSAID. After the second transrectal ultrasound examination, serial examinations were done every 5 minutes for one hour. After 24 hours of NSAID treatments, a third series of transrectal ultrasound examinations was performed to detect remaining post-treatment effects. The EV locations were assigned during ultrasound exam based on Ginther's (1998) methodology, which divided the uterus into 9 segments. Embryonic vesicle movements were compared quantitatively between treatments. The data were analyzed by Kolmogorov-Smirnov test, comparison with ANOVA followed by Tukey test. FM and ML groups induced a 61% and 67% decrease in EV movements per hour, respectively. Unlike FM and ML, FIRO did not interfere with the EV mobility, presenting similar values before and after treatment. As the FIRO is a NSAID COX-2 selective, this result indicated that embryonic and or endometrial prostaglandins are produced predominantly by COX-1. We observed no alteration on EV mobility in all groups 24 hours post-treatments. In conclusion, FIRO was the only NSAID with no effect on EV mobility and is, consequently, safer when used in early pregnant mares.

Keywords: Equine embryo mobility, nonsteroidal anti-inflammatory drugs, prostaglandin

Effect of chronic NSAID administration on ovulation in mares

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Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are routinely used in equine veterinary medicine to manage pain and inflammation. Two studies have recently been published suggesting that administration of NSAIDs to mares in estrus interferes with ovulation. The objective of this study was to determine the effects of chronic NSAID administration on ovulation in mares.

Materials and methods

Quarter Horse type mares (n=18) were evaluated over a total of 24 estrous cycles. Mares were randomly assigned to one of three groups: Group A - control group, Group B - phenylbutazone, and Group C - firocoxib. On Day 8, mares were administered a single intramuscular dose of prostaglandin (cloprostenol, 250 µg) and NSAID treatment was initiated. Nonsteroidal anti-inflammatory drug therapy began with an intravenous "loading dose" (control - sterile saline 8 mLs, phenylbutazone 4.4 mg/kg, firocoxib 0.3 mg/kg) followed by an oral daily "maintenance dose" (control - molasses 30 mLs, phenylbutazone 4.4 mg/kg, firocoxib 0.1 mg/kg) until either ovulation was detected or ovulation failure was determined. Ovulation failure was defined as absence of ovulation within four days after administration of an ovulation induction agent or formation of a hemorrhagic anovulatory follicle. Ultrasound examinations were initially performed every 1 to 4 days based on follicular dynamics. A single dose of the gonadotropin releasing hormone agonist deslorelin acetate (1.8 mg, IM) was administered to induce a timed ovulation. Ultrasound examinations were subsequently performed every 24 hours until either ovulation was detected or ovulation failure was determined.

Results

The ovulation rates were as follows: control 100 % (8 of 8), firocoxib 87.5 % (7 of 8), and phenylbutazone 75 % (6 of 8). Of the mares that failed to ovulate, one mare treated with phenylbutazone formed a hemorrhagic anovulatory follicle while one mare in the phenylbutazone group and one in the firocoxib group ovulated beyond the expected timeframe. There was no significant difference ($P > 0.05$) in ovulation rates between the treatment groups.

Summary

In conclusion, there was no significant adverse impact of chronic NSAID administration during estrus on ovulation in the mare.

Keywords: Hemorrhagic anovulatory follicle, nonsteroidal anti-inflammatory drug, firocoxib, phenylbutazone, equine reproduction

Comparison of blood progesterone values obtained from an in-house one hour enzyme linked fluorescent immunoassay (ELFA) with radioimmunoassay (RIA)

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Introduction

Determination of blood progesterone concentration is routinely performed during equine reproduction case management. The goal of this study was to compare an ELFA progesterone assay (mini VIDAS®; bioMérieux) with a previously validated progesterone RIA.

Material and methods

Experiment 1: Serum, heparinized plasma, and plasma samples (n=54) were collected from eighteen mares. *Experiment 2*: A total of 238 blood plasma samples (EDTA) were collected from 119 mares throughout the estrous cycle. All samples were centrifuged and plasma removed and subdivided into aliquots and frozen. Progesterone analysis was performed using the mini VIDAS® system in accordance with manufacturer's instructions. Briefly, 200 µl of plasma was diluted with 200 µl of serum free buffer; 200 µl of the diluted plasma was deposited into the test cartridge, and the progesterone program was selected. A second aliquot from each plasma sample was analyzed for progesterone by RIA.

Results

Experiment 1: EDTA plasma was determined to be more accurate than serum or heparinized plasma in measurement of progesterone using the mini VIDAS® system (Figure 1). *Experiment 2*: A high correlation (Pearson Correlation Coefficient of r=0.75) for progesterone concentrations was obtained between the mini VIDAS® system and RIA across a wide spectrum of progesterone levels (Figure 2). The inter- and intra-assay coefficients of variation were 9 % and 4 %, respectively.

Discussion

The mini VIDAS® one hour progesterone assay was able to provide similar results to a validated progesterone RIA for equine EDTA plasma. Clinically, the ELFA assay would provide a rapid accurate assessment of progesterone concentration and allow for an early informed decision for reproductive management.

Keywords: Equine, progesterone, analysis, validation

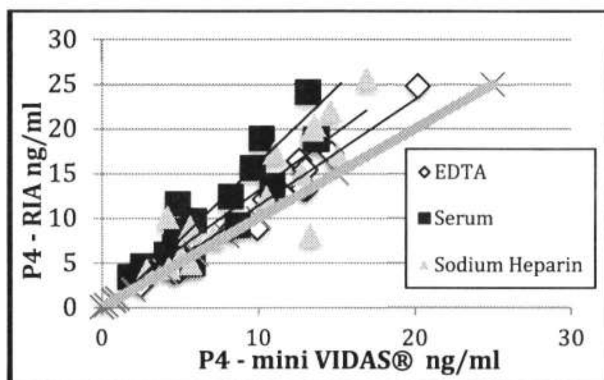

Figure 1. Blood serum and plasma progesterone values obtained by the mini VIDAS® system as compared to RIA. The gray line represents a theoretical perfect concordance between test results.

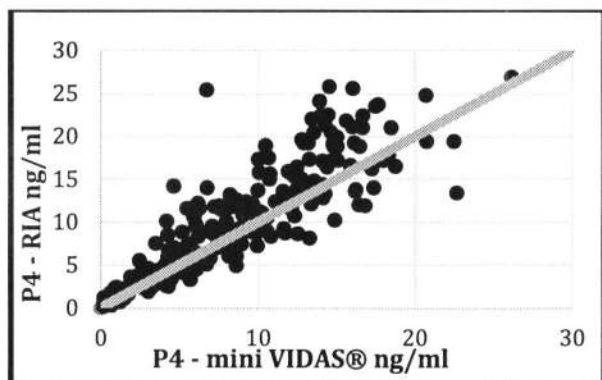

Figure 2. Blood EDTA plasma progesterone values obtained by the mini VIDAS® system compared to RIA. The gray line represents a theoretical perfect concordance between test results.

Qualitative differences in the uterine luminal fluid proteome between normal mares and mares with endometritis or endometrosis

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Comparison of the uterine luminal fluid proteome in mares with a healthy uterus versus mares with uterine disease may help in elucidating the mechanisms underlying normal uterine function and uterine pathology. The objectives of this study were: (a) to evaluate the qualitative differences in the uterine luminal fluid proteome between normal mares and mares with endometritis or endometrosis (chronic degenerative endometrial disease) and (b) to perform a functional classification of the uterine luminal fluid proteome. Uterine luminal fluid samples were collected by low volume lavage from normal mares (n=8) and mares with endometritis (n=23) or endometrosis (n=7). Proteomic analysis was performed using label-free liquid chromatography-tandem mass spectrometry and the data was validated using Scaffold (version Scaffold_4.4.8, Proteome Software Inc., Portland, OR). Proteins that were exclusively present in one of the three groups of mares or common to two or all three groups were identified using the Venn diagram feature available in the Scaffold software. A total of 221 proteins were identified: 147 proteins were common to all three groups; one protein (prostasin) was exclusively identified in normal mares; 39 proteins were exclusively identified in mares with endometritis; 10 proteins were identified in mares with endometritis and mares with endometrosis but not in normal mares; 24 proteins were identified in normal mares and mares with endometritis but not in mares with endometrosis. Functional classification of the proteins using PANTHER (www.pantherdb.org) revealed that 4 proteins exclusively identified in mares with endometritis and 2 proteins common to mares with endometritis and mares with endometrosis were associated with lytic enzyme activity (proteolytic, hydrolytic, or lysozyme activity). The differential presence of these lytic proteins and the absence of prostasin in mares with endometritis and endometrosis may explain, in part, why the uterine environment is not conducive to pregnancy maintenance in mares with these conditions. Although the role of prostasin in equine pregnancy is not yet known, it has been shown to be involved in in endometrial tissue remodeling and trophoblast cell proliferation and invasion during early pregnancy in primates. The results of the present study provide a springboard for future evaluation of specific proteins as potential biomarkers of uterine health and disease and for investigation of their roles in establishment and maintenance of pregnancy.

Keywords: Equine, uterine proteome, endometritis, endometrosis, functional classification

Thawing straws of frozen stallion semen directly in a 37°C water bath versus inside a plastic bag within the water bath

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Introduction

The success of an artificial insemination program relies on proper semen handling techniques. Frozen semen shipments should be accompanied by thawing instructions aimed at optimizing post-thaw motility and, potentially, fertility. Standard thawing instructions must be altered when presented with a cracked or broken straw to prevent exposure of the spermatozoa to water. The goal of this study was to compare post-thaw motility between frozen semen straws immersed directly in a water bath versus frozen semen straws placed inside of a plastic bag and then immersed in a water bath.

Materials and methods

Ejaculates (n=9) were collected from healthy Quarter Horse and Arabian stallions using a Colorado model artificial vagina. The semen was centrifuged, diluted in freezing extender to a concentration of 200 million sperm/mL, loaded into 0.5 mL straws and cryopreserved. Treatment groups were: Group 1 – straws were thawed in a 37°C water bath for 30 seconds; Group 2 - straws were placed into a plastic bag and then immersed in a 37°C water bath for 30 seconds; Group 3 – straws were placed in a plastic bag and immersed in a 37°C water bath for 3 minutes. The latter time period was determined to be the time required for a straw within a plastic bag to reach 37°C as measured by a thermocouple. Sperm motility was evaluated using computer assisted sperm analysis (CASA; SpermVision®, MOFA Global, Verona, WI). Statistical analysis was performed using a one-way repeated measures ANOVA with post-hoc Tukey's test. A significant difference was considered for a p value of <0.05. Data are presented as mean ± SD.

Results

There was no significant difference in post-thaw total sperm motility between the different thawing techniques. Post-thaw total sperm motility for Group 1 was 43.9 ± 17.2 %, Group 2 was 38.3 ± 15.6 % and Group 3 was 38.7 ± 17.1 %.

Discussion These data confirm that there is no significant difference in post-thaw sperm motility if straws of frozen equine semen are thawed in a plastic bag immersed in a 37°C water bath versus the standard technique of direct immersion in a water bath. Consequently, the bag technique can be used if confronted with a clinical situation involving cracked or broken straws of frozen stallion semen.

Keywords: Stallion, frozen semen, cooled storage

Progesterone evaluation in mares: comparison between endocrine laboratories

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An accurate assessment of blood progesterone concentrations is critical for optimal reproductive management of broodmares. The goal of this observational study was to compare progesterone concentrations determined by six different endocrine laboratories in a series of identical plasma samples from mares in various stages of reproduction. Blood samples were collected from nine different Quarter Horse mares at various stages of the estrous cycle. Blood was collected into tubes containing EDTA, centrifuged within 10 minutes (1,000 x *g* for 10 minutes), plasma removed and subdivided into six identical aliquots and frozen at -10°C. Plasma samples were shipped to commercial laboratories on ice using an overnight courier. Four commercial laboratories reported using a radioimmunoassay (RIA) for analysis, while one laboratory used an enzyme immunoassay (EIA) and one laboratory used an enzyme linked fluorescent immunoassay (ELFA). Data are presented as the mean ± SD. Progesterone concentrations for each sample from all six laboratories were within two standard deviations of the mean (i.e. no statistical outliers). However, considerable variation was noted between laboratories for the two samples with the highest P4 concentrations (Sample 1; 8.8±2.8 ng/ml; Sample 3; 6.1±1.9 ng/ml; Figure).

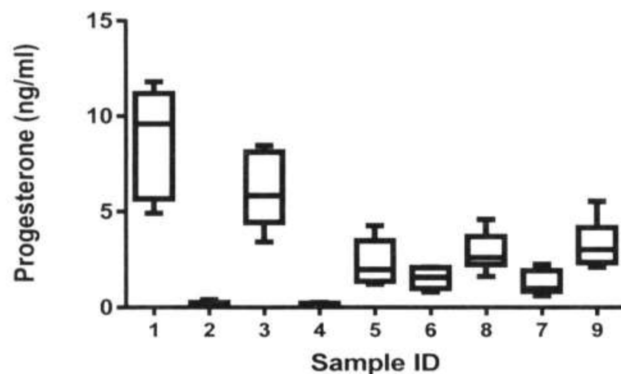

Figure. Progesterone analysis of nine plasma samples by six different endocrine laboratories.

Diagnostically important progesterone values in broodmare management are 1.0 ng/ml, (presence or absence of luteal tissue), and 4.0 ng/ml, (adequacy of luteal function for maintenance of pregnancy). Progesterone values obtained from commercial laboratories are not all identical. There is no uniform standardization between commercial endocrine laboratories.

Keywords: Equine, progesterone, analysis, laboratory

Effects of thawing rate and three extenders on refreezing of equine semen for intracytoplasmic sperm injection

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Intracytoplasmic sperm injection (ICSI) is a reliable technique for assisted fertilization in horses. When frozen semen availability is limited, sperm can be thawed, diluted and refrozen to optimize their use for ICSI. We examined two thawing treatments and three commercial extenders for refreezing stallion sperm. Three 0.5 ml straws of frozen sperm from two different ejaculates were obtained for each of seven stallions; frozen at different facilities and in different extenders. For each ejaculate in the low temperature treatment (LT), one straw was thawed at 37°C for 12 sec (time required for straw to reach 5°C) and rapidly placed in a 5°C water bath. In a 5°C cold room, sperm were divided into three aliquots, diluted 4-fold in the treatment extenders (BOT, Botucrio®, Betlabs, Lexington, KY; EZFMAX, E-Z Freezin® Cryomax™ MFR5; and EZF, E-Z Freezin™ MFR5, Animal Reproduction Systems, Chino, CA) and packaged in 0.5-ml straws. For the room temperature treatment (RT), a second straw was thawed at 37°C for 30 sec and at room temperature (22°C) the sperm divided into three aliquots, diluted and packaged as described. Straws were placed over nitrogen vapor for 15 min, before being plunged into liquid nitrogen. A third straw was used to evaluate original sperm parameters. Sperm were analyzed for total (TMOT) and progressive (PROG) motility by computer-assisted sperm analysis (Sperm Vision®, Minitube, Germany). Percentages of live/dead sperm (LIVE) (LIVE/DEAD® Sperm Viability Kit, Molecular Probes™, Eugene, OR) and sperm DNA fragmentation (DNA) (Sperm Chromatin Structure Assay, Acridine Orange, Sigma, St Louis, MO) were evaluated by flow cytometry. Results are presented as mean±SD. Analyses of variance and least squares means were performed for analyses. After refreezing, all sperm parameters were affected negatively (TMOT: 42±18 and 19±10; PROG: 33±16 and 13±10; LIVE: 37±5 and 10±8; DNA: 5±2 and 8±4; $P<0.02$, frozen and refrozen, respectively). Thawing treatments were also different for TMOT (LT: 23±11 and RT: 14±8; $P<0.001$) and PROG (LT: 17±10 and RT: 9±7; $P=0.002$) with higher sperm motility for sperm thawed only to 5°C. No differences were observed in LIVE (LT: 25±11 and RT: 21±9: $P>0.05$) or DNA (LT: 8±6 and RT: 7±2, $P>0.05$) between thawing treatments. Sperm refrozen in BOT had higher motility (TMOT 23±10; PROG 18±9) than sperm refrozen in EZF (TMOT 14±9; PROG; 9±8, $P=0.02$), but was not different from sperm refrozen EZFMAX (TMOT 19±11; PROG 13±9). The percentage of LIVE and DNA were similar for extender (LIVE+ 22-24%; DNA- 7-8%). In conclusion, refreezing stallion sperm resulted in reduced motility, viability and DNA integrity, compared to sperm frozen only once. However, a modified thawing process, warming the sperm to only 5°C and processing at 5°C before refreezing in both BOT and EZFMAX resulted in sperm with the highest motility parameters, which are sufficient for ICSI.

Keywords: Stallion, semen, freezing, refreezing, thawing, ICSI

Experimental validation of anti-Mullerian hormonae as a quantitative marker of ovarian follicular reserve using unilateral ovariectomy in mares

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Estimates of ovarian reserve have long been sought as predictors of likely fertility. Ovarian follicular hormones are obvious candidates, but most are under feedback regulatory control and normalize despite declines in ovarian follicle numbers under the influence of positive gonadotropic support. However, anti-Mullerian hormone (AMH) is regulated by gonadotropic feedback and has been used as a marker of ovarian follicular reserve in women. To our knowledge, ovarian follicular reserve has not been manipulated experimentally to investigate effects on AMH concentrations, certainly not in horses. Therefore, hemi-ovariectomy was utilized to halve ovarian follicular reserve and AMH concentrations were monitored before and for two weeks after surgery. Fourteen mares of various breeds were hemi-ovariectomized during breeding season under local anesthesia via flank laparotomy. Young (n=6; mean 6.0±0.9yrs) and old (n=8; 18.4±0.7yrs) mares comprised different age groups. The incision was closed in two layers using a continuous suture line incorporating the abdominal muscular layers followed by approximation of the subcutaneous tissue and suturing of the skin. Serum was collected from all mares on the day prior to surgery and daily thereafter for 15 days. Anti-Mullerian hormone was measured using a commercial ELISA kit according to the manufacturer (Equine AMH ELISA, Ansh Labs, Webster, TX). The mean value of AMH before surgery was 1.0±0.1ng/mL and did not differ by age (1.0±0.1 and 1.0±0.2ng/mL, in young and old mares, respectively. Anti-Mullerian hormone concentrations declined progressively thereafter, reaching 0.4±0.1ng/mL 5 days after surgery, 54.5% of pre-surgical concentrations and remained constant thereafter. These data demonstrate that despite the inevitable, progressive loss of ovarian follicular reserve with aging between 6 and 18 years of age, it is not reflected in a significant decline in serum AMH concentrations. However, acute halving of ovarian follicular reserve by surgical hemi-ovariectomy was followed by a decline in AMH to half of pre-surgical concentrations. We conclude that AMH is an acute quantitative, indicator of ovarian follicular reserve after hemi-ovariectomy. However, with the slow progressive decline in primordial follicle populations that inevitably accompanies aging, ovarian compensation and continual replenishment of antral follicle populations obscures this decline in mares up to nearly 20 years of age. Therefore, caution should be exercised in the interpretation of AMH concentrations as a marker of fertility in mares less than 20 years of age.

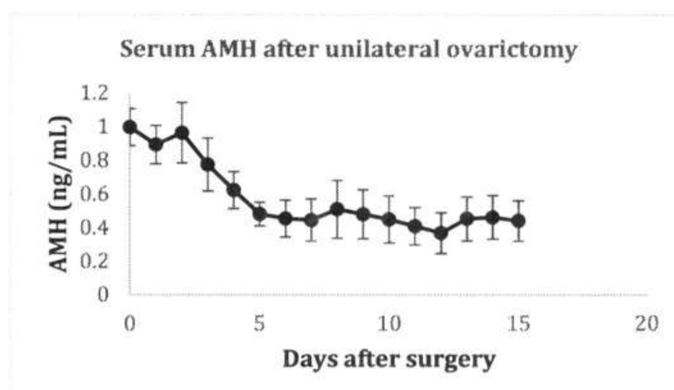

Figure: Daily serum AMH after unilateral ovariectomy surgery.

Keywords: Ovarian follicle, preantral follicle, granulosa cell, ovarian reserve.

Acceleration and deceleration of fetal cardiac frequency during last week gestation of Santa Inês ewes: preliminary results

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The acceleration/deceleration process in human and canine fetuses during the last gestational week has been shown to be an indicator of the ideal moment of parturition. This process begins 72 hours before the birth and intensifies in the last 6 hours. In ewes, these changes have not been described and can be very useful in the prediction of parturition and identification of fetal maturity. This study aimed to evaluate the fetal heart rate (FHR) during the last gestational week and to identify the occurrence of the acceleration /deceleration process in ewes. Fifteen pregnant Santa Inês ewes were evaluated by ultrasonography twice a day during the last gestational week. Fetal heart rate was obtained by the measurement of the pulsed Doppler tracing and this parameter was correlated with the hours before parturition (HAP) by the Pearson test. The acceleration/deceleration phenomenon was identified as positive when, during the 5-minute B-mode visual evaluation, a variation greater than 20% of the fetal heart rate was subjectively observed. The evaluation ranged from 276 to 0.4 (HAP), the HRF was 132.5±16.9 bpm (range 93-203 and 95% CI-130-136 bpm) in this period and did not present a correlation with (p=0.965), single (132.5±17.6 bpm), or twin (132.6±15.0 bpm) were observed in the pre-labor hours (r=-0.106; p=0.166). The fetal heart acceleration /deceleration process was observed in 24 of the 173 evaluations performed (14%), with 67.2±55 HAP with a variation in the FHR between 87-176 bpm. Apparently, fetal heart rate and acceleration/deceleration are not related to the time of parturition in ewes.

Keywords: sheep, labor forecast, ultrasound.

Acoustic radiation force impulse elastography in ovine fetal liver

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Intrauterine fetal tissues development characterization is fundamental to assess fetal viability. We hypothesized that elastography examination will able to detect changes in fetal hepatic tissue stiffness, allowing follow the structural process of organic maturation. Therefore, aiming to evaluate the fetal hepatic tissue stiffness throughout ovine physiologic pregnancy, the fetal liver of 24 healthy pregnant ewes were evaluated weekly, from the tenth week until parturition by qualitative and quantitative acoustic radiation force impulse (ARFI) elastography. The grayscale elastograms were compared qualitatively between gestational weeks (gw) and shear wave velocities (SWV m/s) compared by Friedman's test and correlated with gestational age by linear, quadratic or cubic regression models ($P < 0.05$). At elastogram analysis, the liver presented as not deformable tissue and showed a not variable light gray tone throughout pregnancy. Already, the hepatic SWV (0,79±0,14 m/s at the tenth gw) increased gradually from the fourteenth (0,87±0,22 m/s), reaching it maximum values at the twentyfirst (0,96±0,28 m/s) gestational week ($P < 0.001$) and correlates strongly with fetal age ($R2 = 0.80$). Acoustic radiation force impulse-elastography showed to be an applicable and safe technique that allows detects hepatic stiffness modifications related with fetal development and gestational age.

Keywords: Elastography, fetal development, maturity, pregnancy, ultrasonography

Cervicitis in postpartum dairy cows

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Endometritis has a high prevalence in postpartum cows and its impact on subsequent reproductive performance is significant. In contrast to endometritis, cervicitis has not been intensively studied even thought a negative effect on reproductive performance has been found. The hypothesis of the present study was that the inflammation and/or infection of the cervix and the endometrium are correlated. The first objective was to determine the prevalence of cervicitis in postpartum dairy cows and to determine the association between the vaginal appearance and the cytological assessment of the cervix. The second objective was to evaluate if there was a correlation between inflammation of the cervix and the endometrium. All clinical procedures met the national guidelines for the care and use of laboratory animals and were approved by the institutional animal care committee of the University of Montreal. Three different commercial dairy herds, located in Quebec, were recruited based on convenience. All cows (n = 133) were examined by transrectal palpation and by vaginoscopy between 28 and 42 DIM. The vaginal content and cervical os were visually assessed for the presence of discharge and color respectively. Cytobrush samples from the cervix and the uterus as indicators of inflammation were taken. Data obtained were subjected to descriptive statistics and regression using general linear procedures of statistical software. Cervicitis was found in 32% and 24% of the cows on visual and on cytological evaluation (>5% neutrophils), respectively. Cytological endometritis was diagnosed with 19% of postpartum cows. There was no significant association between the appearance of the cervix and the presence of neutrophils on cervical cytology. However, a positive association ($p<0.0001$) was found between the percentage of neutrophils on cytology of the cervix and the endometrium. Our results suggest that visual and cytological assessment of the cervix could be a simple, but important tool to evaluate reproductive health in postpartum dairy cows.

Keywords: Dairy cows, postpartum, cervicitis

Pharmacokinetics of enrofloxacin and its active metabolite (ciprofloxacin) in late-term pregnant and post-parturient mares – preliminary results

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Antimicrobial therapy is necessary to treat severe bacterial diseases, and ongoing studies in our laboratory suggest that enrofloxacin, a fluoroquinolone antimicrobial with bactericidal activity against gram-negative and some gram-positive pathogens, is an alternative antimicrobial for infections in pregnant mares. Pregnancy induces changes including increases in plasma volume, total body water, renal blood flow, delayed gastric emptying, and hormone-induced changes in hepatic microsomal enzyme activity, resulting in alteration of bioavailability, distribution, metabolism, and excretion of drugs. The changes may necessitate doses and dosing interval adjustments during pregnancy. The objective of this study was to determine the pharmacokinetics of enrofloxacin, administered orally and intravenously, to pregnant and non-pregnant mares using a cross-over design. We hypothesized that the pharmacokinetics of enrofloxacin and its active metabolite (ciprofloxacin) in late-term pregnant mares differ from those of non-pregnant mares. Four light breed, healthy pregnant mares (260 d gestation, n=4) were randomly treated with a single dose of either intravenous (5mg/kg, IV) or oral (7.5mg/kg, PO) enrofloxacin; the other route was given 7d later with both treatments repeated by 45 d postpartum. Plasma samples were obtained at 0, 5, 10, 20, 30, 45, 60, 90 min, and 2, 3, 4, 6, 8, 12, 24, 36, 48 and 72 h after enrofloxacin adminstration (IV or PO). Concentrations of enrofloxacin and ciprofloxacin were measured by LC-MS/MS. Plasma enrofloxacin and ciprofloxacin concentration vs. time were analyzed for each mare based on noncompartimental pharmacokinetics. Normality of the data was assessed using histograms of differences in means, normal quantiles plots of the residuals and the Shapiro-Wilk test. Constant variance of the data was assessed with Levene's test. Comparison of each pharmacokinetic variable between pregnant (PG) and postpartum mares (PP) was done with the paired t test or the Wilcoxon signed rank test ($p \leq 0.05$). After IV administration, apparent volume of distribution and systemic clearance of enrofloxacin were significantly lower in PG than PP mares. For both routes (IV and PO), enrofloxacin showed greater area under the curve concentration vs. time curve ($AUC_{0-\infty}$) and plasma concentration 24h after administration (C_{24h}) in PG mares than PP mares: IV $AUC_{0-\infty}$ 18.3±1.9 (PG) vs 12.8±2.1 µg*h/mL (PP), p=0.03 and IV C_{24h} 0.106 ± 0.035 (PG) vs. 0.032±0.005 µg/mL(PP), p=0.03; PO $AUC_{0-\infty}$ 17.7±4.5 vs 8.2±1.6 µg*h/mL, p=0.03 and PO C_{24h} 0.171±0.078 vs. 0.047±0.015 µg/mL, p=0.049. The ciprofloxacin $AUC_{0-\infty}$ and C_{24h} were larger in late gestation than postpartum period after IV administration, respectively IV $AUC_{0-\infty}$ 3.0±0.7 vs 1.8±0.4 µg*h/mL, p=0.016 and IV C_{24h} 0.036±0.04 vs 0.013±0.002µg/mL, p=0.019. Time to maximum plasma concentration, half life of the terminal phase and oral bioavailability were not significantly different between the groups for all treatments. In conclusion, pregnancy significantly affected the pharmacokinetics of enrofloxacin and ciprofloxacin.

Keywords: Enrofloxacin, ciprofloxacin, pregnancy, mare

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Controlled manual removal of fetal membranes in the horse: a case based application

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Management of the fetal membranes in the postpartum mare is generally directed at management of retained fetal membranes. However, preventative management, specifically controlled manual removal of fetal membranes immediately following parturition, may have added benefits. Cases of on-farm management of high risk (i.e., placentitis) mares, mares following dystocia, or normal foaling mares that are far removed from the practice area may justify early intervention. Once parturition is complete, the mare and foal are allowed to bond. The perineal area is aseptically prepared and the degree of membrane attachment determined through digital examination of the reproductive tract. The procedure can be performed with the mare in sternal recumbency or standing; however, sternal recumbency alleviates the weight of the membranes during removal. The attached portion of the membranes is identified. A scissor-like action of two fingers is used to bluntly "dissect" the most caudally attached portion of the chorioallantois from the endometrium. Once the base of each horn is free from the endometrium in a complete 360-degree area, the membranes can be encircled using the thumb and forefinger. This 'ring' of fingers is used in a gentle manner to move cranially up and back on the attached membranes to evenly separate the chorioallantois from the endometrium. This controlled pressure is repeatedly applied moving cranially while allowing the free portion of the membranes to shift in the palm of the hand. The membranes outside of the mare are also stabilized with the free hand to minimize weight of the detached membranes and prevent tearing or uterine horn eversion. When membranes are edematous from placental pathology it is especially important that the weight of the attached membranes is relieved to prevent uterine horn eversion. In 2016, 18 pony mares were used in a placentitis study at the University of Florida. Placentitis was induced in 12/18 ponies using transcervical placental inoculation of *Streptococcus equi* spp. *zooepidemicus* on the chorioallantois. Six, uninfected mares were allowed to foal normally. Fetal membranes were manually removed in 12/18 mares. Membranes were removed without complications in 9 of 12 mares (9/12; 75%). Three mares (3/12; 25%) suffered complications (uterine horn eversion, prolapse) after membrane removal. Six mares did not undergo manual membrane removal. Membranes were evacuated after dystocia and controlled vaginal delivery under general anesthesia for one mare. One mare delivered her foal prematurely (Day 309 gestation) and retained the tip of the non-gravid horn which was later retrieved. Of the four mares that spontaneously released fetal membranes, 3 mares (3/4;75%) suffered uterine horn eversion while one mare (1/4;25%) had no complications after membranes were passed. All mares suffering complications, independent of spontaneous or manual membrane removal, had placentitis and abnormally heavy fetal membranes (> 11% body weight). Manual removal of fetal membranes was a useful tool in this study. Mares with abnormally heavy placentas were at risk for uterine complications after foaling.

Keywords: mare, fetal membranes, chorioallantois, manual removal, dystocia, placentitis

Acknowledgement

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Management of twin pregnancies by umbilical and fetal oscillation in the mare

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While the use of ultrasonography has reduced twin pregnancies as a major cause of abortion in the mare, diagnosis of twins past the mobility stage still occurs and requires special management techniques. After cessation of the mobility phase, and prior to day 60, options for twin management include ultrasound-guided manual manipulation (ablation or membrane damage), transvaginal fetal puncture (TVFP) and fetal termination by oscillation. Both manual manipulation and TVFP are not always effective and may be difficult or require special equipment. Fetal termination by oscillation, which either alters umbilical blood flow or dislocates the fetus from the umbilicus, is possible between days 45 and 50 of gestation. This stage of gestation is suitable for management by fetal oscillation due to the length of the umbilicus, and was presented as a practice tip at the AAEP Convention in 2013 by this group. It has been performed successfully in cases of unilateral and bilateral cornuate pregnancy fixation. The oscillation technique, without fetal dislocation, utilizes transrectal ultrasonography to image each fetus and monitor heart rate along with umbilical blood flow. Once the fetus to be manipulated is identified and isolated, the linear rectal probe is positioned to allow 0.5 to 2 oscillations per second. Alterations in blood flow can be observed as decreased rate or frequency of blood flow or complete cessation of blood blow. Disruption of fetal blood flow can occur during the first manipulation attempt but may require multiple sessions. The other fetus should be continually monitored throughout the procedure to ensure no negative effects of the oscillation have occurred. The present case series describes the outcome of five 45-50 day gestation twin pregnancies referred to Goulburn Valley Equine Hospital for management from 2014 to 2017.

Unilateral fixation was present in one case while the remaining four pregnancies were fixed bilaterally. Termination of a specific fetus was determined based on uterine position or fetal size. The number of sessions required to alter umbilical blood flow ranged from one to four. The procedure was deemed successful when a heartbeat or umbilical blood flow could no longer be visualized with color flow Doppler ultrasonography. All five cases were discharged with a single viable pregnancy. Normal foals were delivered in 4/5 of the cases described with the fifth mare due to foal in November 2017. No complications were reported during the procedure or throughout gestation. Our rationale for presenting this case series is to provide more information for veterinarians on an effective, noninvasive, management technique for twin pregnancies between 45 and 50 days of gestation.

Keywords: Horse, mare, twin management, post-fixation twins, oscillation

Acupuncture increases matrix metalloproteinase type-2 immunoexpression and tissue concentration in bovine caruncles after calving

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The objectives of this study were to determine the effects of acupuncture in dairy cows on caruncular matrix metalloproteinase type-2 (MMP2) and type-9 (MMP9) immunoexpression and tissue concentration at 0, 2, and 4 hours after calving. The null hypothesis was that acupuncture would not alter MMP expression. Immediately after natural calving, cows were restrained in a stanchion, the perineal area was aseptically prepared, and a caruncle was obtained from the body of the uterus. Half of the tissue was fixed in formalin and the other half was flash frozen. Caruncle collection was repeated at 2 and 4 hours after calving. Acupuncture was administered to cows (n=6) following each caruncle collection (at 0 and 2 hours after calving). Untreated controls (n=9) were kept in a stanchion for 15 minutes without any stimulation. Acupuncture was applied to points reported to relax the cervix and stimulate uterine contractions: bladder 31, 32, and 34, Baihui, Guanyuanshu, and governing vessel-1. Formalin-fixed caruncles were paraffin embedded, sectioned to 6 μm, mounted on poly-l-lysine-coated slides, and subjected to routine immunohistochemistry to determine MMP2 and MMP9 expression. MMP2 and MMP9 immunoexpression was scored by a single observer. Frozen caruncles (0.5 g) were homogenized in buffer and centrifuged. MMP2 and MMP9 concentrations were calculated from caruncular homogenates run in triplicate using commercial bovine ELISAs. The average value (score or concentration) for each time point for each cow was used to calculate the average ± SEM for each treatment group and groups were compared using ANOVA. Significance was defined as $p<0.05$. Immunoexpression of MMP2 and MMP9 was predominately localized to the epithelial and subepithelial stromal cells of the caruncles in both treatment groups. MMP2 and MMP9 immunoexpression was lower four hours after calving in the control cows but not in the acupuncture treated cows, indicating that acupuncture treatment maintained MMP expression. MMP2 tissue concentration was lower two hours after calving in the control cows but not in the acupuncture treated cows and there was a trend ($p=0.09$) for cows treated with acupuncture to have greater MMP2 tissue concentration two hours after calving (after the first treatment) compared to the time of calving. In both treatment groups, MMP9 tissue concentrations were lower at calving compared to two and four hours after calving. This study provides physiologic evidence for the effects of acupuncture on the bovine reproductive tract and substantiates the use of this treatment in cases of placental retention.

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Keywords: Cow, matrix metalloproteinase type-9, placentome, retained placenta

Clinical evaluation of leukocyte esterase as a means of detecting endometritis in the mare

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Clinical endometritis is a leading cause of financial loss in the equine breeding industry. While diagnosis of endometritis is traditionally performed using endometrial culture and cytology leukocyte esterase (LE) has recently been demonstrated to be useful for diagnosing clinical endometritis in dairy cattle.[1] The aim of this study was to describe the results of LE test in a population of forty-eight mares from different farms (44 TB, 1 STB, 1 FRS, 2 QH) with a mean age of 11.27 years (range 3 to 24 years). One mare was tested twice, for a total of 49 subjects. When a veterinarian determined a mare was in estrus, an endometrial swab for aerobic culture and cytology was obtained and processed as previously described.[2] Cytologic evidence of inflammation was classified as: normal/mild (1 to 2 leukocytes/100x field), moderate (3 to 5 leukocytes/100x field), or severe (>5 leukocytes/100x field). After culture and cytology were obtained, a new double-guarded swab (Kalayjian Industries, Inc., Signal Hill, CA) was placed through the cervix and extruded into the uterus. An endometrial scraping was then retrieved using the cap, which was guarded upon withdrawal. The cap was then cut from the pipette into a red-top tube containing 1 mL of sterile 0.9% saline, and the tube was agitated. A Jor-Vet, Vet10 Urine Reagent Strip (Jorgenson Laboratories, Inc., Loveland, CO) was briefly immersed into the sample fluid. The test strip was photographed after two minutes, and the results were blindly recorded using the following manufacturer scale: negative (0 leukocytes/μL), trace (15 leukocytes/μL), small (70 leukocytes/μL), moderate (125 leukocytes/μL) or large (500 leukocytes/μL). This test has not been validated as a method of identifying leukocytes in equine uterine fluid, but studies in cattle have shown a high correlation between the presence of uterine inflammation and positive LE test results (96% sensitivity 98% specificity).[3] Of the mares evaluated, 21% (10/48) had positive LE test results (6 trace, 2 small, 1 moderate, 1 large). All six mares with trace LE results had inflammation, and three of the six had positive cultures. Both mares with small LE had severe inflammation but negative cultures. The moderate and large LE mares had severe inflammation and positive cultures. Of the thirty-nine mares with negative LE results, thirty-five were negative for both inflammation and culture growth. The other four mares consisted of two with inflammation but negative cultures, and two others with positive cultures but no inflammation. This study describes the use of the LE test as an aid in diagnosing inflammation associated with endometritis in the mare. Further investigation is necessary to confirm this assay as a stall-side test for endometritis.

Keywords: Endometritis, mare, leukocyte esterase assay, cytology

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Evaluating the efficacy and safety of silicone O-ring intrauterine devices as a horse contraceptive through a captive breeding trial

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Due to continued on-range population growth there are upwards to 70,000 feral horses against a BLM range ecologist established goal of 27,000. Therefore, the need for an effective means of contraception has significantly increased. A contraceptive that is reversible would allow for the preservation of genetic diversity in targeted populations and alleviate some of the concern over contraception in wild horses. In this study we evaluated the efficacy and safety of an O-ring intrauterine device (IUD) based on earlier studies indicating a greater than 80% retention rate. With this background information we hypothesized that a silicone O-ring IUD with a durometer grade (hardness) of 50 would be retained within the uterus and would be capable of preventing pregnancy in mares under normal pasture breeding conditions. Acceptable rates of loss for the IUDs were pre-determined to be not more than 25% during the first month and less than 50% during the three-month trial period. If these parameters were not met the study would then transition to determining "best" fit. Additionally, we also wanted to determine the incidence of adverse reactions to IUDs regarding inflammation, endometrial fibrosis, and cyclicity. The first IUDs inserted had a loss rate of 3/5 over a two month period without stallion exposure. Therefore, five groups of IUDs, of differing sizes and durometers, were manufactured and the second group of O-ring IUDs were inserted into the uterus of 20 young, reproductively healthy mares; the mares were immediately turned out with stallions after IUD insertion. Retention of IUDs was monitored via transrectal ultrasonography at 3-5 day intervals. Biopsy samples were collected at the time of IUD insertion and IUD removal for comparison. After the first 45 days of the pilot study the inserted O-ring IUDs experienced an overall loss rate of 60%, with some IUD configurations having a loss rate of 100%. At that point the IUDs with the lowest rate of loss were selected to be further refined and inserted into a larger group of mares. Three durometer types were inserted in three groups consisting of eight mares each. This step tested the hypothesis that higher durometer IUDs would be less malleable to uterine contractions (during estrus and during the process of breeding) and less likely to be expelled. Over the one-month evaluation period that followed, 11/24 IUDs were lost; many of which were expelled within the first week after insertion. There was no significant increase in endometrial inflammation or fibrosis. Several mares did experience deviations from normal cyclicity. Finally, no pregnancies were established.

Keywords: Intrauterine device, feral horse, O-ring, endometritis

Using behavior to time initiation of oxytocin administration to prolong luteal function in mares

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Poor performance and undesirable behavior during estrus are common complaints made by horse owners and trainers. Administration of exogenous oxytocin starting on Day 7 of diestrus has been shown to be effective in extending luteal function and preventing estrus behavior in mares. The current therapy protocol requires serial veterinary examinations to determine the exact date of ovulation, which may not be feasible in all situations. The objective of this study was to use estrus behavior alone to determine the appropriate time for starting the oxytocin protocol. We hypothesized that administration of oxytocin beginning 8 days after the onset of behavioral estrus will prolong the luteal phase. Twenty-two light breed mares (aged 4 to 20 years) were teased once by introducing them to a stallion and observing for signs of sexual receptivity. Mares not displaying signs of estrus received 250 ug of cloprostenol (IM) and were teased again 3 to 4 days later. On the day that estrus behavior was observed (Day 0), mares were randomly divided into two groups: Oxytocin (n=11): oxytocin (60 IU, IM) was administered once daily from Day 8 to 17; Control (n=11): did not receive treatment. Blood samples were collected from all mares every 4 days throughout Day 17, and every 7 days thereafter until Day 45. Serum progesterone concentration was determined by chemoluminescence and progesterone concentrations >1 ng/ml were indicative of a functioning corpus luteum. Interestrus interval was defined as the period between Day 0 and the day when serum progesterone reached <1 ng/mL. The average interestrus interval between groups was compared by independent samples t-test. Data are presented as mean ± SEM. Significance was set at $P<0.05$. The average interestrus interval was higher for oxytocin treated mares compared to control mares (21.5±1.6 vs. 32.4±4.2 days, respectively). In the oxytocin group, the interestrus interval was longer than 31 days in 6/11 (54.5%) mares and up to 45 days in 5/11 mares (45.45%). Our results are supportive of our hypothesis but are slightly lower than reported success rates obtained in previous studies that initiated oxytocin treatments based on the day of ovulation (60 to 70% response rate).[1] Potentially, the success rate of our study would be improved if oxytocin treatment duration were extended beyond 10 days. The same authors reported improved efficacy when oxytocin administration was extended up to 29 days.[1] We conclude that luteal maintenance was attained by once daily oxytocin administration beginning 8 days following behavioral signs of estrus.

Keywords: Oxytocin, mare, estrus, behavior, corpus luteum

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Morphological evaluation of the stallion spermatozoa through three staining methods

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Evaluation of sperm morphology is part of the assessment of fertility in human or animal reproduction. The analysis can be performed using different techniques, including the use of staining methods. In our prospective study, morphology of the sperm of the horse was evaluated using three staining methods: Diff Quik® (Siemens Healthcare Diagnostics Inc., Deerfield, IL), Eosin-Nigrosin® (RAL Diagnostics, Martillac, FR) and Spermblue® (Microptic Automatic Diagnostic Systems, Barcelona, SP), a stain used for human sperm. Our hypotheses were that (1) Spermblue® would allow an easier reading of the sperm morphology of the horse and allow a better identification of sperm abnormalities, (2) there would be differences in classification of sperm morphology depending on the evaluator's experience. Semen was obtained from various breeds of horses. Forty samples from stallions between 2 and 15 years of age were collected during the 2016 breeding season and stored in a 2% buffered formaldehyde solution until processed. For each sample, three semen smears were made and stained with Diff-Quik®, Eosin-Nigrosin® and Spermblue®. All morphological parameters were then evaluated blindly using a light microscope by a novice evaluator and a more experienced evaluator. For each slide, 200 spermatozoa were examined randomly and were classified into eleven categories according to morphology: normal, abnormal acrosome, abnormal head, detached head, abnormal midpiece, bent midpiece, proximal droplets, distal droplets, abnormal tail, bent tail, round cells and others. Statistical analysis was performed using linear mixed model; staining and observer were within-subject factors. There were no replicate experiments. Independently of the staining methods used, there was no significant difference between evaluators in the identification of morphologically normal spermatozoa. In contrast, significant differences between evaluators were observed in the classification of some anomalies affecting mainly the midpiece and the tail. A poor fixation of the dye was also observed with Spermblue®.

Keywords: Stallion; sperm morphology; staining methods; Spermblue

Expression of maturation-associated genes in equine cumulus cells during in-vitro maturation is affected by the ratio of oocyte-cumulus complexes to medium

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Little information is available on the factors affecting oocyte quality after in vitro maturation (IVM) in the horse. In vivo, expansion of the cumulus cells (CC) and ovulation is dependent on the expression of amphiregulin (AREG), epiregulin (EREG), prostaglandin endoperoxide synthase 2 (PTGS2) and protein-induced tumor necrosis factor 6 (TNFAIP6). In horses, to date only PTGS2 has been identified in the CC during IVM. We hypothesize that health and function of CC are altered by the density of cumulus-oocyte complexes (COC) during IVM. Our objectives were to measure AREG, EREG, PTGS2 and TNFAIP6 mRNA levels and rates of apoptosis in equine CC after culture of COCs at different densities. In Exp. 1, equine COCs were cultured in maturation medium at 5 COC/ 50 μl; AREG, EREG mRNA levels were evaluated by qPCR at 0, 1, 2, 6 and 30 h. In Exp. 2, COCs were cultured at 3, 5, 10 or 20 COC/50 μl. The mRNA levels for these genes were measured after 2 h, and degree of apoptosis in CC was measured by annexin-V staining after 30 h. AREG and EREG mRNAs were upregulated at 2 h of culture. In Exp. 2, levels of PTGS2 ($p=0.002$) and TNFAIP6 ($p=0.04$) mRNA were significantly upregulated only at low COC density, whereas AREG and EREG mRNA levels were not affected by COC density. COC density had no influence on the degree of cumulus apoptosis after 30 h of culture. In conclusion, this study demonstrates that COC density may have an impact on cumulus cell function in a commercial IVM setting. The use of a culture medium with low COC density may improve the expression of the genes involved in the expansion of CC.

Keywords: Horse, oocytes, in-vitro maturation, cumulus cells, genes.

Management of extreme form of uterine torsion in a doe: a case report

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A two year old mixed breed doe was presented to the ISU Theriogenology Service with a history of prolonged duration of gestation (> 1 week) and labor (> 36 hours). At presentation the doe was bright, alert, and responsive and displayed no signs of ongoing labor. There was no sign of vulvar discharge nor were there any fetal membranes or structures visible upon physical examination. A speculum examination revealed the caudal portion of the vaginal tract twisted counterclockwise leading to a suspicion of a possible uterine torsion. An abdominal ultrasound exam revealed a single fetus with absence of a heartbeat. A cesarean section was performed and revealed an extreme form of uterine torsion (>360 degrees) resulting in an ischemic uterus and severely congested and dilated uterine vasculature. A complete blood count intra-operatively revealed a low PCV of 14%. A single large dead fetus (full term) was extracted from the uterus and it was untwisted. It took three complete clockwise rotations to return the uterus back to its original position and axis. The dilated uterine vasculature was traced individually and ligated, and a complete ovariohysterectomy was performed. The doe recovered uneventfully after surgery and was then managed medically for her low PCV and other derangements of her blood parameters. Postoperatively, the anemia was addressed via a whole blood transfusion and the doe was started on a regimen on intramuscular procaine penicillin G. Pain was managed with intravenous flunixin meglumine and intramuscular butorphanol. Forty eight hours postoperatively the doe was found to be pyrexic (temperature 104.6 F) and was managed with intravenous flunixin meglumine, topical isopropyl alcohol application, and a fan for evaporative cooling. Investigations into the cause of anemia excluded gastrointestinal nematodes, abomasal ulcers, as well as hemolytic events, and it was hypothesized that the marked, diffuse venous dilation of the uterus and broad ligament, as noted on uterine biopsy, with subsequent blood trapping was a contributing factor to the anemia. McMaster's examination yielded a moderate *Eimeria sp.* burden, and the doe was treated with sulfadimethoxine. She was discharged fourteen days after surgery, and had a full recovery to her previous role as a showmanship animal. Uterine torsions are a relatively uncommon occurrence in goats with incidences higher in does carrying a single fetus. Clinical signs range from non-productive labor, abdominal discomfort and constant straining. The present case differs from usual presentations, with the patient showing no apparent outward clinical signs considering the extreme degree of uterine torsion. Such torsions lead to sequestration of blood in major vascular beds such as the uterine arteries leading to acute anemia. Moreover, detorsion of the affected organ can lead to reperfusion injuries and further derangements in the cardiovascular status of the patient. Prognosis for acute long-standing, extreme uterine torsions is poor with regards to the future health and breeding prospects of the affected animal owing to ischemic damage to the uterus. The present case highlights these aspects as well as the successful surgical and medical management leading to a favorable outcome in terms of patient health.

Keywords: Torsion, doe, anemia, extreme, uterine

Hydrops and unusual placentation in a mare: a case report

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A 20 year old multiparous Quarter Horse broodmare in the eighth month of gestation presented to the Iowa State University Equine Theriogenology service with a history of progressive abdominal distension causing discomfort and hind limb lameness. The mare had previously been diagnosed and was being treated for equine pituitary pars intermedia dysfunction (PPID) with pergolide mesylate. She also had an incidental non-symptomatic first degree AV block. The mare's vital parameters were within normal limits; however the mare seemed uncomfortable and depressed upon physical examination. Based on the presenting signs, a tentative diagnosis of fetal hydrops was made. To confirm this diagnosis, the mare was evaluated by trans-rectal palpation, trans-rectal ultrasound, and trans-abdominal ultrasound. Trans-rectal examination revealed a fluid-filled distended turgid organ at the pelvic brim that revealed hypoechoic fluid with swirling particulate matter. No fetal parts or normal reproductive tract could be palpated. Upon trans-abdominal ultrasound, a live fetus with a heart rate of 96 bpm could be identified in the caudal abdomen. At the level of the fetal thorax, an oval mass 17 cm in diameter containing hypoechoic fluid could be identified. Excessive allantoic fluid could be seen further confirming a diagnosis of hydrops allantois. Due to the advanced age of the mare, previous fertility problems and prior parturitions, and health issues of the mare (PPID, arrhythmia), the owner elected for humane euthanasia. Postmortem examination confirmed the hydrallantois diagnosis with 40 gallons of yellow fluid evacuated from the allantoic sac. A 32 cm cyst-like structure was found within the amniotic sac adjacent and contained two gallons of bright yellow, slightly viscous, flocculent fluid. The surface opposed to the amniotic membrane was bright red and appeared similar to the chorioallantois. There was abundant, small, friable white to tan particulate material within the fluid and on the inner surface of the cyst-like mass. Microscopic examination of the fetal membranes revealed mineralization, edema and splitting of the allantoamnion, the latter corresponded with the fluid-filled sac appreciated during gross postmortem examination. The microscopic changes described within the uterus are likely within normal range for a multiparous, aged mare. Mineralization of the placenta can occur and is not likely of clinical significance. The fetus had a crown-rump length of 57.7 cm and appeared normal for the stage of gestation. Hydrallantois is a relatively uncommon complication of pregnancy in equines. The cause of hydrops is unknown but abnormalities in fluid production and absorption, vascular abnormalities and possibly genetic factors are thought to play a role. Hydrallantois typically leads to very dramatic abdominal distention are at risk of prepubic tendon rupture or abdominal herniation due to stress placed on the abdominal wall and foals of affected mares are often abnormal and may have torticollis or other abnormalities.

Keywords: Hydrallantois, equine, hydrops, placentation

Lactational anestrus in Caribbean donkeys

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Lactational anestrus is a common postpartum finding in many species of animals, but is uncommon in equids. While horses are seasonally polyestrous, the majority of donkeys ovulate year round, even at temperate latitudes (43°N) although duration of estrus and interovulatory interval are sensitive to season. Conversely, horse mares are sensitive to season even in temperate climates such as southern Mexico (latitude 15–22°N). Almost all horse mares ovulate soon after parturition, with a very small minority entering an anovulatory period most often associated with foaling in the non-ovulatory season. This case report reviews an unusual finding of eight donkeys of a group of twenty-five on the Caribbean island of St. Kitts (17°N) entering anestrus after foaling during the spring and summer months. Twenty-five feral donkeys were obtained from the island of Nevis and transported to the sister island of St. Kitts to participate in institutional research at Ross University School of Veterinary Medicine. Pregnant jennies were required as an inclusion parameter for the study, and all were confirmed to be late in gestation via trans-abdominal ultrasonography. Each jenny was between the ages of three and thirteen years, in good body condition, and foaled without complications between March and July 2016. Jennies and foals were monitored daily postpartum for uterine involution, nursing and general health. Estrous cycles were evaluated using trans-rectal ultrasonography of ovarian structures beginning one week after parturition and continued at weekly intervals. Estrus behavior was detected using fertile jacks twice weekly. Four foals experienced neonatal disease that resulted in fatal septicemia; their dams were monitored closely and found to cycle normally following the loss. The remaining twenty-one jennies had healthy foals at their sides for four to six months before weaning. Thirteen jennies experienced a foal heat with detectable signs of estrus within two weeks of giving birth, then returned to normal cyclicity. However, eight of the jennies did not ovulate or show signs of estrus until their foals were weaned approximately four months later. After weaning, all eight donkeys promptly returned to normal cycling. The jennies were all housed together in an outdoor pasture, received the same nutrition, and were in similar body condition, with no conspicuous loss of condition within the herd. Although we can attribute the lack of return to normal estrus following parturition to factors such as shortened daylight hours, disease, or nutritional deficits in equids, all seem unlikely in this instance. In many species lactation and nursing of offspring can suppress follicular growth and block leutinizing hormone, thus inhibiting ovulation. The fact that each jenny returned to a normal estrous cycle following weaning supports the hypothesis that eight of the twenty-five donkeys entered lactational anestrus during the physiological breeding season, a hitherto unreported phenomenon. However, blood progesterone assay's from each donkey would be needed to confirm a true case of lactational anestrus.

Keywords: Donkeys, Caribbean, reproduction, lactational anestrus, estrous cycle

Clinical findings in a warmblood mare following intrauterine infusion of the commercial preparation of enrofloxacin (Baytril®)

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Baytril,® a commercially manufactured suspension of enrofloxacin, has been demonstrated to cause severe inflammation and degeneration of the endometrium when infused into the uterus of mares.[1] However, little information is available regarding the management and future fertility of these cases following infusion. This abstract describes the history and clinical findings in a mare that received a single intrauterine (IU) infusion of Baytril®. A 17-year-old warmblood mare was presented for reproductive evaluation with the following history: Culture of an endometrial swab yielded growth of *Enterobacter cloacae* and a single IU infusion of an undisclosed quantity of Baytril® was administered. Severe endometritis occurred following infusion, and several uterine lavages recovered hemorrhagic and fibrinous exudate. Following this treatment the mare was bred once unsuccessfully with cooled-shipped semen, and at 15 days post-ovulation transrectal ultrasound revealed a small amount (<0.5cm in the dorso-ventral plane) of anechoic fluid in the uterine lumen. 48 days after the initial Baytril® infusion the mare was referred for reproductive evaluation. Transrectal palpation and ultrasonography revealed active ovaries, no uterine edema, <1 cm of anechoic luminal fluid, and a moderately toned cervix. Digital evaluation of the cervix revealed no abnormalities. Hysteroscopy demonstrated numerous circumferential, white, fibrous adhesions coursing diffusely over the surface of the endometrium. Only one small (<2 cm wide) transluminal adhesion was seen located at the base of the left uterine horn and extended from the two to seven o'clock position. Loss of the normal endometrial architecture was further evidenced by linear and punctate ulcerative lesions spread in a multifocal pattern from base to tip of both horns. Histologic evaluation of samples collected from the endometrium revealed a category IIB biopsy with mild diffuse lymphocytic infiltrate of the stratum compactum, mild fibrosis circumscribing individual glands (one to three layers) and three glandular nests per 5 mm linear field. A second biopsy performed 60 days later was a category III with significant increase in the amount of fibrosis present, three to five layers of fibrocytes circumscribing individual glands and five glandular nests per 5 mm linear field. Based on the severity of these findings, it was determined that existing conventional treatments would be unlikely to normalize the endometrium adequately to support pregnancy, even following prompt aggressive therapy.

This case summarizes the history and clinical findings in a mare following Baytril® infusion and highlights the benefit of hysteroscopy as a diagnostic tool to determine the extent of damage following caustic infusions.

Keywords: Enrofloxacin, mare, hysteroscopy, endometritis

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Ejaculate quality evaluation and freezing ability in a hemi-castrated stallion following spermatic cord torsion

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Spermatic cord torsion occurs occasionally in stallions and often represents a surgical emergency. The effect of the condition on sperm quality is not well described. In the present clinical case, we describe ejaculate quality characteristics and sperm freezing ability of a stallion following 360° spermatic cord torsion and hemi-castration.

The stallion underwent unilateral castration surgery after an acute 360° spermatic cord torsion episode. Semen collection, evaluation and freezing ability were evaluated 2 months (collection 1) and 7 months (collection 2, 3 and 4) after surgery. Ejaculate parameters assessed included volume, sperm concentration, total spermatozoa per ejaculate, motility using computerized assisted sperm analysis (SpermVision®, Mofa, Verona, WI), morphology using two different stains (eosin/nigrosin and Spermac®) and viability using sybr-14 and PI under fluorescent microscopy. To determine freezing ability, three semen freezing protocols (E-Z Freezing "LE"®, E-Z- Freezing "MFR5"® and Botu-crio®) and two centrifugation techniques (INRA 96® and Equi-Pure™) were used. Not all techniques were used simultaneously. Methods and results per ejaculate are shown in the table. The most common head abnormalities observed in morphology evaluation were pyriform head, knobbed acrosome, and macro- and microcephalic spermatozoa. No differences were obtained in morphologic evaluation between E/N and Spermac®.

In conclusion, ejaculate quality may take several months to improve after severe spermatic cord torsion and hemicastration. Fresh semen quality improved significantly starting two months after surgery, however severe sperm abnormalities and poor freezing ability was observed. It is not known what mechanisms are involved in delayed recovery of freezing ability. The effect of spermatic cord torsion and hemicastration on spermatogenesis, membrane integrity and sperm chromatin stability merits further studies.

Keywords: Spermatic cord torsion, hemi-castration, fertility

Table: Ejaculate parameter results

	Collection 1	**Collection 2**	**Collection 3**		**Collection 4**
Total motility (%)	35	70	67		70
Progressive motility (%)	33	65	46		50
Morphology (Eosin/Nigrosine) (%)	35	55	37		40
Normal	26	15	25		12
Head defects	14	3	21		17
Proximal droplet	-	24	17		31
Mid piece defects					
Centrifugation medium	INRA 96®	INRA 96®	INRA 96®/ Equi-Pure™)		INRA 96®
Freezing Method	E-Z Freezing "LE"®	E-Z Freezing "LE"®	E-Z Freezing "LE"®	E-Z- Freezing "MFR5"®	Botu-crio®
Post-thaw motility (%)	5	25	24	28	25
Viability in fresh semen (%)	-	52	77	78	70

Collection of stallion using XL human latex condom

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A twelve-year-old Quarter Horse stallion was presented for inability to ejaculate. Prior to presentation, the stallion had been collected using a Missouri AV by the owner. The stallion had been trained to mount a breeding phantom and mount mares in standing estrus as well as ovariectomized mares at the owner's farm. Approximately one month prior to presentation the stallion became unwilling to ejaculate in the Missouri AV. The owner reported he would mount the mare but would stop thrusting when his penis was placed in the Missouri AV.

Initially, upon presentation, the stallion was willing to mount an ovariectomized jump mare and ejaculate in the Missouri AV. Approximately two weeks from arrival he stopped mounting the ovariectomized jump mare but continued to mount the mare in standing estrus. As the weeks progressed he became more difficult to collect. Five times he required multiple mounts. A number of changes were made to ensure an ejaculation. Imipramine (1000 mg) was given orally two hours prior to collection. The ovariectomized mare was replaced with a mare in standing estrus. The possibility that pain was causing the unwillingness to ejaculate was also considered. He was placed on phenylbutazone (4.4 mg/kg) PO BID for 10 days and 6 grams ranitidine PO BID for 30 days. Subsequently, he was placed on 57 mg firocoxib PO SID. He was also allowed pasture turnout on a daily basis.

Upon the suggestion of a theriogenologist, an XL latex human condom (Trojan Magnum XL Latex Condom, Princeton, NJ) was used in place of the Missouri AV. The stallion was allowed to tease to a mare in standing estrus while in his stall. The mare was then placed in the collection area and the stallion was brought to the mare and allowed to tease to full erection. Once the stallion was fully erect, he was moved away from the mare and a technician placed the XL latex condom on the end of his penis, extending approximately 1/3 of the length of his shaft. The stallion was allowed to go back to the mare and mount. The stallion entered her vaginally, thrusted, and ejaculated. As the stallion dismounted, the technician removed the condom with the ejaculate. The ejaculate was processed routinely for cooled semen transport and acceptable pregnancy rates were achieved. On rare occasions, the condom would break during ejaculation, but collection was achieved 2 hours later. The stallion would not mount an ovariectomized mare, but he would mount mares in standing estrus. The imipramine was discontinued without any negative consequences.

This abstract illustrates the use of a human latex condom to collect an ejaculate from a stallion that had developed the inability/aversion to ejaculate into a Missouri AV. This method of collection provided a reliable method of obtaining an ejaculate when numerous other modalities had been attempted and it was imperative to obtain semen to satisfy breeding contracts and salvage a breeding season.

Although not a routine collection method, use of a XL latex condom is effective for stallion collection.

Keywords: Equine, collection, latex condom

Diagnosis and surgical removal of uterine masses in two mares

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Uterine neoplasia is rare in the mare with a limited number of reports in the literature. Tumors that have been described affecting the endometrium include fibrosarcoma, fibroma, adenocarcinoma, lymphosarcoma, leiomyosarcoma, fibroleiomyoma and rhabdomyosarcoma. The most commonly described uterine neoplasia in mares is leiomyoma, a benign neoplasia arising from the outer smooth muscle of the uterus.[1] This abstract describes the diagnosis and removal of uterine masses in two mares. The masses were very similar when visualized via trans-rectal ultrasound, however were determined to be of different origin on histopathology. Mare A, a 15 year old Thoroughbred mare was presented for examination of a 6 cm diameter mass at the cranial tip of the right horn. The mare had foaled approximately 45 days earlier and the mass had first been identified approximately 25 days after foaling on routine examination for breeding. The mass was palpaable as a firm solid structure and trans-rectal ultrasonic examination revealed a 3.5 x 5 cm intraluminal mass with a soft tissue echogenicity. Hysteroscopic examination revealed that the mass was pedunculated and attached to the dorsal wall by a thin, 1 cm diameter stalk. The cervix was manually dilated and the hand advanced up the right horn until the mass could be grasped. Via manual manipulation the mass was peeled off of the endometrium and removed from the uterus with minimal hemorrhage. Histopathologic examination identified the mass as a uterine stromal polyp, a benign, non-neoplastic, idiopathic proliferation of the endometrium, more common in older mares. Mare B, a 20 year old Friesian mare, was presented for evaluation of a uterine mass that had been identified during trans-rectal ultrasound examination for routine breeding management. Trans-rectal ultrasound examination revealed a 7 cm mass of soft tissue echogenicity at the base of the left horn. Hysteroscopic examination revealed that the mass was attached to the dorsal uterine wall by a broad based, 1 x 3.5 cm, stalk. An attempt to remove the mass manually was unsuccessful due to the thickness of the stalk and significant hemorrhage from the stalk after manipulation. A vessel sealing device (LigaSure™; Medtronic, 710 Medtronic Parkway, Minneapolis, MN) was utilized to provide hemostasis and remove the mass. Histopathologic examination identified the mass as a uterine leiomyoma. Uterine leiomyomas are a relatively common tumor of the reproductive tract, are generally slow growing and do not metastasize. This abstract describes successful removal of two different types of uterine tumors that were indistinguishable via trans-rectal ultrasound, hysteroscopically or grossly.

Keywords: Uterine neoplasia, uterine mass

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Use of oxytocin to prevent return to estrus in a mare after breeding

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A seven year-old multiparous Thoroughbred mare being managed for live cover failed to become pregnant on two subsequent cycles despite the use of routine breeding management. The mare had a body condition score 4.5/9, adequate perineal conformation and was bred to a Thoroughbred stallion with known fertility. Ultrasonography examinations for pregnancy were conducted on Day 14 after ovulation. At the time of each pregnancy examination there was no ultrasonographic evidence of a visible corpus luteum, intrauterine fluid, or an embryonic vesicle. The mare was displaying signs of estrus with a moderate degree of uterine edema (grade 2/3; 0-none, 1-mild, 2- moderate, 3- severe), a dominant follicle >=35mm and a relaxed cervix. Uterine culture and cytology were performed after each pregnancy examination. There was no bacterial growth or cytologic evidence of infection or inflammation which would account for the early return to estrus. Furthermore, there was no evidence of an iatrogenic cause for early return to estrus. Based on the clinical findings at the time of each pregnancy examination and the inability to identify a cause for early return to estrus, it was presumed that the mare underwent premature luteal regression. The mare was naturally served a third time by the same stallion and similar breeding management was utilized. In addition, the mare was administered a daily injection of oxytocin (60 units, IM, Q24hr) from Days 7-14 after ovulation, as oxytocin has been shown to maintain luteal function in cycling mares.[1] Transrectal ultrasonography performed on Day 14 after ovulation revealed a positive pregnancy result; furthermore, a visible corpus luteum was present, the cervix and uterus were toned and there was no evidence of uterine edema. Follow-up examinations conducted on Days 17, 30, and 45 after ovulation confirmed normal embryonic growth and development. The author concluded that although the positive pregnancy result may have been possible without the use of oxytocin, the administration of oxytocin from Day 7-14 after ovulation may be beneficial in maintenance of early pregnancy.

Keywords: equine, luteolysis, oxytocin, pregnancy maintenance

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Time of breeding can influence cria gender ratios in alpacas

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The control of gender for economic gains in livestock production has been widely investigated, and anecdotally unsuccessful. Production gender ratios are generally left to chance, except for usage of commercial sperm sorting or other invasive and costly reproductive technologies. The objective of this randomized prospective study spanning six years was to investigate the role of hand breeding alpacas at certain times of day to achieve preconceptual gender selection. A total of 92 hembras from two production farms located in the Northeast region of the United States were included in this study regardless of age, parity, or previous cria's gender. Hembras were randomly assigned to 1 of 2 breeding seasons, either spring/summer (May-August) n= 39 or fall (September-November) n=53. They were then tested for receptivity and each was hand bred at a random time on the day of male acceptance, from 7:00am-7:00pm. Location, time of breeding and gestational length was recorded for each hembra, and subsequent cria gender was confirmed at parturition. Breeding times were consolidated into three groups in each breeding season, <9:00 am, 9:00 am-4:30 pm and >4:30 pm based on natural breaks in gender ratios. Data were analyzed for effects of time of breeding on gender ratio using Fisher's exact and odds ratio statistical methods. Gestational length did not differ for male or female gestations (345 vs 343 days). Additionally, there was no effect of location of breeding, and thus implied management variability, on gender ratios ($p>1.0$). However, results revealed that cria gender ratios were altered by time of breeding irrespective of breeding season ($p<0.05$). For spring/summer season, breeding at or before 9:00 am resulted in 100% females (n=10), compared to breeding between 9:30 am-4:30 pm which resulted in 46% females and 54% males (n=11 and 13, respectively). Although a smaller subset, a dramatic shift occurred with breedings after 4:30 pm, resulting in only 1 female and 4 males. Similarly for the Fall breeding season, 74% female cria resulted from breedings prior to 9:00 am (n=22), followed by 50% females and 50% males breeding between 9:30 am and 4:30 pm (n=28). Only 1 breeding occurred after 4:30 pm during Fall, presumably due to shorter daylight lengths, and that single breeding resulted in a male cria. Results of this study are the first report describing a simple managerial practice to alter gender ratios in alpacas. Breeding early in the morning favored selection for female gestations, whereas breeding in evenings during this study resulted in more male cria. Interestingly, midday breedings tended to result in gender ratios similar to that observed on both farms prior to initiation of this study with combined 53% females and 47% males recorded for over 300 births. Given the lengthy gestations in alpacas, being able to preconceptually control gender could revolutionize the alpaca industry in this country, both in terms of economic growth and also increased textile development through larger production herds.

Keywords: alpaca, cria, gender ratio

Use of the prostaglandin E1 analog misoprostol to hasten oviductal transport of equine embryos

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Non- surgical collection of small equine embryos for cryopreservation is recommended at day 6.5 to 7 after ovulation. Collection at day 7 or later will yield large embryos which are not suitable for standard cryopreservation techniques; however, embryo recovery rates are lower for day 6.5 to 7 than for day 7 and later. In horses, the embryo secretes prostaglandin E2 (PGE2) around day 5 after ovulation. This embryonic hormone acts locally into the oviduct, stimulating rapid movement of the embryo and its entry into the uterus some 24 h later. Local administration of PGE2 hastens oviductal transport of equine embryos, but involves abdominal surgery and is unpractical for the sole purpose of recovering small embryos for cryopreservation. Misoprostol is a potent synthetic prostaglandin E analog and can be administered orally, but may affect progesterone production by the corpus luteum as PGE is involved in several luteal pathways. Hypotheses: We propose that the treatment of early pregnant mares with oral misoprostol on day 5 after ovulation will hasten oviductal transport of equine embryos thus increasing recovery rates at day 6; and will also affect progesterone levels.

Objective: To compare the time of embryo recovery (day 6 vs. day 6.5 after ovulation) and progesterone levels for mares treated with two doses of oral misoprostol versus untreated controls.

Materials and methods: Fifteen cycling mares of mixed light breeds, 3 to 17 year-old, were used. Following detection of endometrial edema and a preovulatory (>35 mm) follicle, ovarian activity was monitored twice daily and mares were artificially inseminated with $\geq 0.5 \times 10^9$ motile spermatozoa every other day until ovulation was detected (ovulation day = day 0). Bred mares were randomly assigned to the untreated control group or misoprostol group. Misoprostol was given orally at 0.009 mg/kg BW, on days 5 and 5.5 after ovulation. On day 6 after ovulation, standard non-surgical embryo recovery was attempted; if an embryo was not recovered, a second attempt was carried out on day 6.5 after ovulation. The time at which the embryo was obtained in each group for each mare (paired samples) was the end point for the statistical analysis; therefore, only mares that yielded an embryo in both groups were included in the analysis. After the initial randomized assignment, mares alternated experimental groups until an embryo was recovered, then she was assigned to the other group until the second embryo was recovered. Cycles with unilateral double ovulation were excluded. Blood samples were taken from the jugular vein at 12 h intervals form day 5 to day 6.5, progesterone concentrations were determined by RIA, and results were analyzed as repeated measures and compared as LSM.

Results: eight mares yielded an embryo in both experimental groups; 7 out of the 8 embryos were recovered on day 6 and the remaining embryo was recovered on day 6.5 in each group (different mares). Progesterone concentrations were not significantly different ($p \geq 0.05$) for control vs. misoprostol groups (12.5 ± 1.3 ng/ml vs. 11.8 ± 1.2 ng/ml, LSM ± SEM). In conclusion, we were not able to accept or reject the hypothesis that misoprostol hastens oviductal transport of equine embryos; however, we showed that oral misoprostol at the given dose does not affect progesterone concentration. In this experiment, the recovery rate at day 6 did not significantly improve by waiting 12 more hours in contradiction with traditional literature; further research is needed in this area.

Keywords: Equine, PGE, misoprostol, embryo recovery, oviductal transport

Equine cooled-semen shipping container effectiveness comparison

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The objective of this project was to determine the effectiveness of cooled-semen shipping containers by comparing total and progressive motility after 24 h of cooled storage in the containers at ambient temperature. The three container types included Equitainer™ (ET; Hamilton Research, Inc., Ipswich, MA), Equine Express II™ (EE; Exodus Breeders Corporation, York, PA), and EquiSaver (ES; Platilite Corporation, Omaha, NE). Two ejaculates were collected on separate days from two Thoroughbred stallions (15 and 19 y.o.) with recently documented fertility. Each ejaculate was evaluated for concentration, total motility, and progressive motility using a computer assisted semen analysis system (SpermVision SAR®, MOFA Global, Verona, WI). The semen was diluted with Revolution™ extender with gentamycin (Reproduction Resources, Inc., Walworth, WI) to a concentration between 25-50 x 10^6/mL. Each extended ejaculate was divided into nine, 5 mL aliquots in polystyrene tubes wrapped with parafilm. The tubes were placed in 50 mL centrifuge tubes, surrounded by 22°C water. One aliquot of each stallion's semen was placed in each of the nine shipping containers, three containers from each of the three companies, along with a temperature data logger (Digit-TL LabJack®, Lakewood, CO), which recorded temperature readings every 10 minutes. After 24 h storage between 21 and 23°C, each aliquot was evaluated for total and progressive motility. Mixed effects linear regression was used to model the effects of time and shipping container company on total and progressive motility measures, and temperatures inside of the containers; post-hoc comparisons were adjusted using a Bonferroni correction (Stata IC/13.1, StataCorp LP, College Station, TX).

The 24 h total motility and progressive motility were similar in all aliquots, regardless of container (P=0.86 and P=0.94). The temperatures of the containers at 24 h ranged from 1.9°C to 16.1°C depending on the company (average temperatures: ET 15.8°C, EE 7.6°C, and ES 2.4°C). There were significant differences in temperatures detected between all three companies at 24 h ($P<0.05$). Overall while there were significant differences detected in the internal temperatures between the shipping containers, there were no significant differences in the effectiveness of these containers keeping semen motile for 24 h while the containers were stored at room temperature. The next step would be to test the containers in more extreme conditions. Further studies should determine how wide the range of temperatures could be inside the containers before motility is significantly affected. Since only one extender was used, future studies should also evaluate the effect of the extender on the cooling rate and final temperature in each shipping container.

Keywords: Stallion, cooled semen, shipping containers, sperm motility

Management of a high-risk pregnancy in a Portuguese Water Dog

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A 5-year old female Portuguese Water Dog was presented with a history of infertility and early pregnancy loss followed by one pregnancy that produced three offspring with intensive monitoring. The patient was bred this cycle on days two, three and four after ovulation.

At 33 days gestation, pregnancy was confirmed by transabdominal ultrasonography. The serum progesterone concentration this day was 12 ng/ml. At 40 days gestation, the serum progesterone concentration decreased to 7.2 ng/ml. It further declined to 5.3 ng/ml at 43 days and 2.3 ng/ml at 46 days. Progesterone concentrations are highest at gestational days 20 to 35, reaching concentrations of 15 to 80 ng/mL.[1,2] A sustained decrease in serum progesterone <2 ng/ml for greater than 24 hours will result in pregnancy loss.[3,4] Use of progesterone supplementation during gestation may lead to masculinized females and cryptorchid male puppies; therefore, supplementation should be postponed to day 50 of gestation or used only when risk of pregnancy loss is imminent.[3,4] Due to steadily declining progesterone concentrations, the patient was started on oral altrenogest, a supplemental progesterone (ReguMate®, 0.088mg/kg orally once daily) at 43 days gestation.

Additionally, the pregnancy was monitored using the Whelpwise® tocodynamic monitoring system. Terbutaline was administered starting on day 46 following documented uterine contractions. Transabdominal ultrasound was performed regularly throughout gestation to monitor fetal health and development.

At 57 days gestation, she was admitted to the hospital. Altrenogest was slowly decreased and terbutaline dosage was increased slightly due to increasing uterine contractions. At 61 days gestation, ultrasound showed good intestinal motility and well-defined fetal kidneys. Fetal heart rates decreased to 150-180 bpm and a cesarean section was performed. Six viable puppies were delivered. This case represents how to successfully manage a high-risk pregnancy in a bitch with a documented history of hypoluteoidism while maintaining fetal and maternal health.

Keywords: Progesterone, ultrasound, high-risk, pregnancy

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Successful medical management of long term pyometra in a Boston terrier bitch

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A three-year-old intact female Boston terrier was referred to the Veterinary Medical Teaching Hospital at UC Davis with a 25 day history of intermittent vaginal discharge and reluctance to run agility as usual. She had received antibiotics and intravenous fluids from the referring veterinarian, and was hospitalized briefly at her local veterinary clinic after an episode of collapse, vomiting, and fever.

On presentation the bitch was bright, alert, and responsive, with vitals within normal limits. Ultrasound revealed a fluid-filled uterus (1.5 cm) with significant thickening of the uterine wall (1 cm) and evidence of cystic changes. The bitch was a valuable breeding animal, so medical management was elected over ovariohysterectomy. Treatment was performed using a cloprostenol and cabergoline protocol that minimized the dose of prostaglandin necessary. Multiple rechecks showed improvement of the pyometra. The evident cystic changes resolved over time and six months later she was successfully bred by trans-cervical insemination and carried two healthy puppies to term.

Traditionally, the treatment of choice for pyometra is ovariohysterectomy, but medical treatment using progesterone receptor antagonists (aglepristone and mifepristone), prostaglandins (dinoprost and cloprostenol), dopamine agonists (cabergoline), or different combinations of these drugs have been reported with success.[1-5] Reported success rates vary greatly, as the studies are small and medical management has generally been reserved for either cases with mild to moderate pyometra or severely affected bitches where surgery is not a viable option (including cases with peritonitis).

This case demonstrates that medical management of pyometra can be a successful alternative to ovariohysterectomy, even in cases of long duration, allowing for preservation of fertility in reproductively valuable females.

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Nocardioform placentitis in three Thoroughbred mares in Kentucky

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Nocardioform placentitis is a preeminent cause of late term reproductive losses in Kentucky broodmares,[1] with a sharp increase in cases during the 2017 breeding season. The following cases illustrate usual presentation, recommended diagnostics, treatments, and potential outcomes.

A six-year-old maiden, a 21-year-old multiparous, and a seven-year-old mare with her second pregnancy from the same Thoroughbred farm were presented at 8, 9, and 10 months gestation, respectively, for premature mammary development. On ultrasound the first two mares showed placental edema, thickened amnion with a closed cervix transrectally, and placental uterine separation transabdominally. Treatment included daily antibiotics, non-steridal anti-inflammatory drugs, pentoxyphilline, and altrenogest. Antibiotic choice was based on current research in nocardiform bacteria sensitivity.[2] Ultrasound evaluation of the mare presenting at 10 months was within normal limits; as a precaution a similar treatment regime was initiated. Variable outcomes were seen. The mare presented at 8 m aborted at 301 D. The mare presented at 9 m delivered an unthrifty foal at 309 D which was later euthanized. The mare presented at 10 m delivered a normal viable foal at 323 D. Gross pathology of the chorion was classic in all three cases and showed an avillous area at the base of the horns or body covered by a brown mucoid exudates.[3,4] Histopathology showed blunted chorionic villi. Uterine cultures were all negative. Culture and cytology from the edge of the chorionic lesion from the viable foal were positive for nocardioform bacteria.

Nocardioform bacteria are environmentally ubiquitous but, despite active research, the source and route of transmission are not understood.[5] Classic clinical signs include premature mammary development, no vaginal discharge, and separation of placenta noted transabdominally, but not transrectally. In these three cases, the onset of clinical signs progressively later in gestation correlated with more favorable outcomes. This potential correlation may direct initial discussion with clients of probable prognosis.

Keywords: Nocardioform bacteria, placentitis, abortion, pregnancy loss, broodmare

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Reproductive failure associated with the co-infection of porcine circovirus type 2 and porcine reproductive and respiratory syndrome virus

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Spontaneous porcine abortions and stillbirths have a significant economic impact; however, the lesions are usually not pathognomonic and a definitive etiology is often identified in only one-third of abortion cases.[1] The recently developed multiplex polymerase chain reaction (PCR) used in this case may be a convenient diagnostic tool for rapid and simultaneous detection of abortifacient pathogens, especially for the case of co-infection.[2]

Increased incidence of late-term abortions, stillbirths and premature farrowings occurred in a 450-sow, farrow-to-finish, closed farm in Northern Taiwan from November 2016 to January 2017. Abortuses and stillbirths from six litters were necropsied. Gross lesions included cardiomegaly, congested liver and meninges, patchy pulmonary hemorrhage, and pleural and abdominal effusion in the affected fetuses. Histopathological examination revealed mononuclear myocarditis (1 of 6 litters), meningitis (2 of 6), and vasculitis (1 of 6) in some litters. Few protozoa-like organisms were detected in the myocardium (3 of 6). Co-infection of porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) was demonstrated in two litters by multiplex PCR, sequencing and immunohistochemistry. Infection of PCV2 was found in two other litters. Specific lesions and etiologies were not identified in the remaining two litters. Other abortifacient viruses, including classical swine fever virus, encephalomyocarditis virus, Japanese encephalitis virus, Menangle virus, porcine parvovirus, and pseudorabies virus, were ruled out by the negative result of PCR.

There is no recommended treatment for reproductive failure associated with the co-infection of PCV2 and PRRSV. To eliminate non-immune animals, vaccination of all gilts and sows for PCV2 prior to breeding is recommended since vaccination of only gilts with lower immunity level against PCV2 may not be sufficient.[3,4] None of the current vaccines can completely prevent PRRSV infection,[5] but vaccination of gilts and sows can improve the reproductive performance,[6] which potentially reduces economic loss.

Keywords: Abortion, co-infection, porcine circovirus type 2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV), stillbirth, swine

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Surgical resolution of a papayraceous mummified fetus in a 5 year old Brahman cow

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A 5-year old Brahman cow used for oocyte and embryo collection presented for a suspected mummified fetus after a prolonged gestation of 397 days. Physical examination indicated an asymmetrically enlarged uterus with a 7 month fetus.[1] The remainder of the examination was unremarkable. Transrectal ultrasound, transabdominal ultrasound, and electrocardiography were unsuccessful in detecting a fetal heartbeat. A BioPRYN serum pregnancy test also indicated the cow was not pregnant.[2]

A recumbent ventral midline hysterotomy was performed to remove a large papayraceous mummified fetus without complications. Two milliliters of oxytocin was administered intramuscularly post-operatively. The cow was discharged twenty days following hysterotomy.

Fetal mummification can be caused by a variety of infectious, genetic, or metabolic conditions that can occur during gestation and result in fetal death.[1] Fetal dessication and absorption of the placental and fetal fluids occurs, and the uterine walls pull close against the mummified fetus. A corpus luteum and closed cervix are present, and the cow may have a prolonged gestation if not identified earlier via palpation. Papayraceous mummified fetuses, those with parchment thin skin and absence of putrefaction, are the most common type.

Treatment of most mummified fetuses is by administration of dinoprost tromethamine and manual extraction of the fetus. Hysterotomy is performed when dinoprost tromethamine is unsuccessful. The ventral midline approach is preferred for a large mummy.[3,4]

For many cases of mummified fetuses, the cow will be culled due to the cost and loss of pregnancy. Medical and surgical treatment can be pursued for valuable cows. Ventral midline hysterotomy when performed under strict aseptic techniques can result in both the removal of the mummy and successful return to fertility.[3,4] This case illustrates the value of good surgical technique when using hysterotomy to remove a mummified fetus, and the emerging value of electrocardiography in determining fetal viability.[5]

Keywords: Fetal electrocardiography, fetal mummification, pregnancy-associated glycoprotein, bovine

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Azoospermia in a stallion caused by a sperm granuloma

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The etiology of azoospermia in the stallion includes ejaculation failure,[1] occluded ampullae,[2] retrograde ejaculation,[3] sperm granuloma,[4] or primary testicular dysfunction.[5] A 3-year-old Gypsy Horse stallion presented to the Texas A&M College of Veterinary Medicine for a breeding soundness evaluation. This stallion's semen had been collected 8 months prior to admission and was found to be azoospermic. At presentation, physical examination was normal with the exception that the stallion was a unilateral cryptorchid. There was no history of illness or trauma to the scrotal testis. Two ejaculates were collected in an artificial vagina, but no sperm were identified in the semen. During both semen collections, the stallion demonstrated good libido and appeared to ejaculate normally (~8 urethral pulses/ejaculate; semen alkaline phosphatase >3000 U/L), indicating that fluid from the testis/epididymis was present in the ejaculate and that an occluded ampulla was unlikely.[5,6] The bladder was catheterized to determine if retrograde ejaculation occurred, but no sperm were identified. The scrotal testis and epididymis were examined manually and by ultrasonography. No testis abnormalities were identified and the testis volume was 78 cc. However, a firm mass (~2 cm) was identified in the vicinity of head of the attached epididymis. Testicular biopsy was considered to rule out a primary testicular problem. Because of this stallion's limited breeding value, the owners elected to have the stallion castrated bilaterally. Histopathology revealed normal spermatogenesis in the scrotal testis and a sperm granuloma in the head of the associated epididymis. Sperm granulomas can result from congenital (e.g., blind-ended efferent ductules) or acquired etiologies (e.g., trauma) and are more common in bulls, goats, and rams than stallions.[7,8] The stallion in this report was young and had no history of trauma or illness; therefore, a congenital cause was considered most likely.

Keywords: Stallion, azoospermia, sperm granuloma

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Vaginal leiomyosarcoma following history of pseudopregnancy in an Anglo-Nubian goat

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Importance

Reoccurring pseudopregnancy in goats can lead to vaginal leiomyosarcomas due to the prolonged progesterone elevation. This is a risk that should be evaluated in caprine patients presenting with pseudopregnancy.

Diagnostic approach and treatment

An 11 year-old Anglo-Nubian doe was presented for bloody discharge visualized from her vulva. She had a history of pseudopregnancy for approximately eight years. Two years previously, she was presented with a swollen left teat. Transabdominal ultrasound revealed an enlarged, fluid filled uterus and a lactating left teat. At that time, an ovariohysterectomy was recommended but the owner declined and elected to monitor for any changes in her condition. At presentation in November 2016, she was down, lethargic, vocalizing, and had frank blood coming from her vulva. She passed a blood clot from her vulva two days prior to presentation. An 8-centimeter mass on the right ovary was diagnosed on transabdominal ultrasound. The packed cell volume was 11%). Euthanasia was elected by the owner at that time, and a necropsy was performed.

Results and discussion

The necropsy revealed a vaginal leiomyosarcoma and a thymoma in the cranial mediastinum. By histology, thymoma, leiomyosarcoma, moderate chronic teat sinus ectasia, and moderate acute centrilobar hepatic necrosis were diagnosed. The vaginal neoplasia was mistaken for an ovarian mass on ultrasound. The anemia was caused by the acute blood loss of the vaginal leiomyosarcoma. The liver necrosis was caused by hypoxia from the anemia. Older goats commonly have incidental thymomas. The incidence of pseudopregnancy in goats ranges from about 3 to 20%.[1] Pseudopregnancy is a condition where aseptic fluid collects in the uterus (hydrometra) and a persistent corpus luteum is present causing elevated progesterone.[2]. The elevation in progesterone can lead to leiomyosarcoma.

Keywords: Doe, vaginal, leiomyosarcoma, pseudopregnancy

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Management of urine accumulation within the preputial cavity in a 2 year-old Holstein bull

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Importance

Immediate attention to preputial injuries is important to prevent infection and fibrosis forming a stricture of the prepuce. This case report describes the diagnosis of preputial fibrosis and stenosis causing urine pooling, resulting in poor semen quality.

Diagnostic approach and treatment

A 2 year-old Holstein bull was presented for persistent swelling of his caudal prepuce. Five months prior to presentation, he had been found hanging with his rear legs over a gate at a bull stud facility. Over time, a persistent swelling appeared at the caudal aspect of the prepuce. The bull was collected but the ejaculates were contaminated with urine and the penis was not exteriorized. On presentation, the prepuce had swelling over the caudal half. Ultrasonography revealed fluid accumulation. Palpation of the preputial orifice revealed fibrosis and stenosis that nearly obstructed the entire opening. The final diagnosis was trauma induced fibrosis causing urine accumulation in the preputial space. Surgery was recommended to attempt to remove as much of the fibrosis as possible.

Results and discussion

Under general anesthesia, the fibrotic area was exteriorized through the preputial orifice and slowly dilated. Once sufficiently dilated, an endoscope was introduced in order to visualize the caudal internal prepuce and glans penis. The fibrotic area was incised in several areas throughout the circumference in order to further dilate the stricture. The glans penis was extended and a preputial reefing was performed. Four days post-operatively seroma of the sheath was diagnosed with ultrasound. Six-days post-operatively, one third of the ventral/left aspect of his incision dehisced. These complications were managed with anti-inflammatories and icing. The bull was discharged 14 days post-operatively. Seventy days post-operatively, the bull was reexamined. He had fibrotic stricture similar to the original presentation. A second surgery was recommended. However, due to financial considerations, the bull went to slaughter.

Keywords: Urine, preputial cavity, preputial resection and anastomosis

Bilateral hydrosalpinx with elevated anti-mullerian hormone in a 5 year-old Belgian mare

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Importance

To date, there have been no reports of hydrosalpinx causing increased levels of anti-mullerian hormone (AMH). This case demonstrates a misdiagnosis of granulosa-theca cell tumor based on ultrasound and blood AMH levels, with a true diagnosis of bilateral hydrosalpinx upon abdominal laparoscopy.

Diagnostic approach and treatment

A 5 year-old Belgian mare was presented in April 2015 for a pregnancy examination. The owners had just purchased her with a history of pregnancy from a February 2016 breeding. Transrectal ultrasound revealed a non-pregnant uterus and with a 60 X 57 mm anechoic structure suspected to be on the left ovary and a 59 X 60 mm anechoic structure suspected to be on the right ovary. Due to the large size of the structures, it was difficult to confirm their tissue of origin. Deslorelin acetate (1mL IM) was given. Transrectal ultrasound rechecks over the next two weeks revealed that those structures only increased in size (>70mm X >70 mm bilaterally) despite additional deslorelin acetate (2 ml IM) administration. Blood AMH levels were 8.2 ng/mL (reference range: 0.1-3.8 ng/mL). The owner elected an exploratory laparoscopy in order to further evaluate the reproductive tract. A standing abdominal laparoscopy performed in September 2016 revealed both ovaries appeared grossly normal, with unremarkable fine-needle-aspirate results. However, the oviducts were enlarged and fluid filled (192mL and 142 mL aspirated from the right and left, respectively.) Cytology was consistent with cystic fluid with mild lymphocytic inflammation. Under laparoscopy, dinoprostone gel (approximately 1gm) was applied topically on the oviducts bilaterally.

Results and discussion

Two weeks post-operatively, the right oviduct appeared to be within normal limits but the left oviduct was fluid-filled on transrectal ultrasound. January 2017 examination findings were consistent with a cycling mare and bilateral fluid-filled oviducts. The owner is currently contemplating whether to pursue *in vitro* fertilization or salpingo-oophrectomy.

Keywords: Hydrosalpinx, anti-mullerian hormone, mare

Acutely fast growing ovarian sex cord-gonadal stromal tumor in a 13-year-old American Quarter Horse mare

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Importance

Granulosa cell tumors have been described by ultrasound to vary in shape ranging from uniformly dense masses with one or many large fluid filled cyst like structures, however, a single fluid filled granulosa-theca cell tumor (GTCT) that acutely doubles in size within 48 hours has never been reported in a mare.

Diagnostic approach and treatment

A thirteen-year old American Quarter Horse mare was referred to the University of Tennessee for evaluation of a right ovarian mass. Earlier in the breeding season (April 2016) the mare was on altrenogest and had an intrauterine marble placed by the referring veterinarian to decrease behavioral changes coinciding with estrus. Upon presentation, the right ovary was unable to be visualized entirely or measured on ultrasound due to its large size (approximately 10 cm diameter on palpation). The presumed ovarian mass appeared to be a single hypoechoic cavity. The left ovary had multiple small follicles present and the uterus was unremarkable except for the intrauterine marble. Differential diagnoses included GTCT, persistent follicle, or hemorrhagic follicle. At that time, altrenogest was discontinued and 1 mL of deslorelin acetate was administered IM. Over the next two days the right ovarian mass enlarged to approximate 25 cm. On ultrasound, the structure appeared to be a single hypoechoic cavity with areas of increased opacity. Blood was submitted for a hormonal profile. Surgical removal of the right ovary was recommended due to the rapid growth of the mass and potential subsequent rupture.

Results and discussion

A 25 cm X 30 cm mass was removed via a ventral midline incision. The fluid filled structure ruptured during removal. It was submitted for histopathology. The hormonal profile was suggestive of GTCT (anti-Mullerian hormone: 18.9 ng/mL). Histopathological diagnosis was sex cord-gonadal stromal tumor, consistent with a GTCT.

Keywords: Mare, ovary, tumor, fast growth

Leptospira induced abortion in 18 year old Thoroughbred mare

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An 18 year old Thoroughbred mare was presented at 286 days gestation with vaginal discharge and enlarged udder. On vaginal speculum examination, there was approximately 15 ml of tan colored fluid within the vaginal vault, and the cervix was softening. A culture of this fluid was negative for bacteria. Serum total progestin and estrogens were normal for her gestation stage. With a tentative diagnosis of placentitis, treatment with altrenogest, trimethoprim-sulfa, and flunixin meglumine was initiated.

The mare returned at 292 days gestation. A small area of placental separation was noted on transrectal ultrasound and the fluid pooling in the vagina was confirmed to be urine based on specific gravity and creatinine levels. Fetal heartrate was 80 bpm. Treatment was continued.

That night she aborted. The placenta was intact with an area of demarcation at the cervical star corresponding to the placental separation. Giant multinucleated hepatocytes were noted on necropsy.

Based on the foal's necropsy findings, the mare was tested for leptospirosis. Very high titers were found for *Leptospira interrogans* serovar *pomona*. Three other pregnant mares on the farm were tested with paired serum samples and titers were low.

While the standard for leptospirosis diagnosis is culture and identification, in abortion cases, high titers in maternal serum are considered diagnostic.[1,2] Streptomycin and penicillin are the antibiotics of choice, with penicillin G used to treat pregnant mares with high titers to prevent fetal infection.[1,2] Leptospirosis is often subclinical in horses and can contribute to reproductive disorders, including abortion and breeding failure.[2,3] Because it can persist in the genital and urinary tracts, detection and treatment is important in breeding populations.[2]

The mare's titers were significantly reduced two weeks later. Because titers were decreasing, treatment was not initiated as the infection did not appear active. The mare was bred three months later and is currently pregnant.

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Severe mating injury in a British White heifer

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Breeding injuries in heifers are not well documented. Young heifers housed with adult bulls are prone to injuries due to their size and may present under different complaints/clinical signs. We report here, an unusual presentation of a breeding injury in a young heifer.

A 2 year old British White heifer presented to WSU-VTH with a history of straining to defecate, urinary incontinence, abnormal ambulation, and anorexia. She was pastured with several cows and three bulls, all appearing normal. The heifer was quiet, alert, and responsive. Physical parameters were normal. Severe swelling, edema, and cellulitis was noticed in perianal area involving the right vulvar labium. A caudal epidural was administered. Transrectal palpation revealed a normal uterus and ovaries but severe edema and excessive straining inhibited palpation of the cervix and caudal vagina. Transrectal ultrasonography demonstrated a normal uterus, static right ovary and mature corpus luteum on the left ovary. A heterogeneous (43x24mm) mass with abscess-like appearance was observed in the vestibular vagina along with hypoechoic area in the dorsal aspect of the vagina, caudal to the bladder. A vaginal speculum could not be introduced beyond the vestibule. Dorsal-lateral tears were present in the vestibule and a large tear containing a friable mass was visualized at the vestibular sphincter. A flexible endoscope was introduced through the vulva to evaluate lesions. The urinary bladder was catheterized, allowing visualization of a communication with the dorsal vestibular tear.

A breeding injury was suspected. We hypothesize that a congenital anatomical malformation of the vestibulo-vagina contributed to the severity of the lesions. A poor prognosis for fertility was given and the owner was advised to cull the heifer. This case illustrates the importance of a complete clinical evaluation of the reproductive tract in heifers presenting with tenesmus, ill thrift, and abnormalities of the perineal area.

Habronemiasis in a 20-year old gelded American Pony

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A 20-year old gelded pony presented with paraphimosis and a swollen, distended prepuce first observed two months previously. A 12 x 20 cm ulcerated mass was located along the internal and external lamina of the preputial fold. Differential diagnoses included neoplasia (squamous cell carcinoma) and habronemiasis. Histopathology of a 10mm punch biopsy of the preputial mass was consistent with habronemiasis. No evidence of neoplasia was present.

Musca domestica (house fly), is a common intermediate host for the stomach worm, *Habronema muscae*. Habronemiasis is the result of the dead or dying larvae of *Habronema muscae* causing a hypersensitive reaction.[1,2] Clinical signs consist of proliferative granulomatous tissue are most often found on the prepuce, external genitalia, ventral abdomen, and limbs.[2] Treatment options include surgical debulking, topical or systemic corticosteroids to reduce inflammation-associated tissue proliferation, fly control, topical preparations containing larvicidal, antimicrobials, anti-inflammatory ingredients, and cryotherapy.[1,2]

Due to location and size of the mass on the prepuce, surgical management was elected. A partial phallectomy and en bloc preputial resection was performed. Moderate post-operative hemorrhage occurred from the corpus spongiosum at the surgical site. Hemorrhage persisted post-operatively during and immediately following urination. A temporary perineal urethrostomy was performed to allow healing of the corpus spongiosum at the site of phallectomy. Additional treatments included intravenous aminocaproic acid, intravenous fluids, flunixin meglumine, 1% diclofenac sodium gel, gentamicin, phenylbutazone, procaine penicillin G, trimethoprim-sulfamethoxazole, and moxidectin-praziquantel gel. The prognosis for this patient is good, and swelling and hemorrhage continued to decrease while in the hospital.

This case is significant because it illustrates the importance of proper diagnostics when approaching a preputial mass. Proper fly control and prevention is important to protect from reproductive loss due to habronemiasis. It also is a good illustration of the diagnosis and surgical treatment of preputial habronemiasis.

Keywords: Habronemiasis, penis, prepuce, phallectomy, mass

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Exposure to exogenous estrogen as a cause of hyperestrogenism

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Hyperestrogenism is a condition generally associated with an increase in endogenous estrogen caused by dysfunction in the hypophyseal-pituitary-gonadal axis, frequently the result of a granulosa or sertoli cell tumor. However, it is important to consider exposure to exogenous estrogen when examining patients with symptoms resembling an estrogen-secreting gonadal tumor.

Pandora, a 2.5-month-old intact female Pit Bull, was presented with significant perineal enlargement and a history of urine dribbling and straining to defecate. A digital examination revealed no perineal hernia; normal urethral and vestibular anatomy was visualized on vaginoscopy. A vaginal cytology revealed cornification of the epithelial mucosa. Differential diagnoses at that time included an estrogen-secreting gonadal tumor, a disorder of sexual development (suspected from the distorted appearance of the external genitalia), or exposure to exogenous estrogen. Diagnostic options included abdominal ultrasonography to identify the gonadal architecture or presence of a tumor, karyotyping, or a baseline hormone panel. Initial treatment options included tramadol to reduce discomfort associated with the vulvar swelling and surgical removal of the gonads.

Taking into account the identified cornification of vaginal epithelium in a pediatric patient, a more comprehensive discussion with Pandora's owner was pursued and revealed that the client was being treated under the guidance of her doctor with Evamist®, an estradiol transdermal spray used to treat vasomotor symptoms consequent to menopause. The owner was applying the product to her arms for a duration of two weeks before the onset of Pandora's clinical signs.

Pandora's case illustrates the importance of obtaining a comprehensive history from clients in order to prevent unnecessary diagnostic tests and procedures. Pandora's owner went on to change the Evamist® application site from her arms to her abdomen. Roughly a month and a half later, Pandora's swelling decreased and Pandora continues to do well today with no recurrence of her clinical signs.

Keywords: Hyperestrogenism, canine, exogenous estrogen

A challenging case of ampullary blockage in a geriatric American Saddlebred stallion

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A 21 yr-old stallion was referred for inability to mount the phantom and subsequent ejaculatory failure during four sequential mounting attempts. Upon presentation, the stallion achieved erection but refused to mount the phantom. Transrectal ultrasonography revealed distended ampullae with hyperechogenic material in the glandular lumen, consistent with ampullae blockage. Bilateral hindlimb suspensory ligament degeneration and orthopedic pain, localized to the pelvis and/or hip, were the primary physical limitations to mounting. Therapy for the orthopedic pain included a combination of shockwave therapy, electro-acupuncture, firocoxib (56 mg PO), gabapentin (5 mg/kg PO) and corrective shoeing. On two occasions, ampullae massage, oxytocin (20U, IV) and chemical induction of ejaculation (2,000mg imipramine PO and 250mg xylazine IV) were attempted but this was unsuccessful. Multiple attempts at collection on the ground and with the phantom were performed for four days with vigorous ampullae massage, imipramine (1,000 mg PO) and oxytocin prior to collection. On day 5, the stallion successfully mounted the phantom and ejaculated. The filter contained tan material consistent with semen plugs and the sample consisted mostly of detached sperm heads. Collection was repeated twice daily on subsequent days using a combination of teasing, imipramine, and oxytocin. The first five ejaculates resulted in necrospermia and teratozoospermia containing 20-30 billion sperm. From ejaculates six to eleven, the percent of teratozoospermia decreased from 85% to 45% and asthenozoospermia decreased from 90% to 50%. Screening for antibodies with flow cytometry revealed 56% IgA-bound spermatozoa and 1.7% IgG-bound spermatozoa. IgA binding could explain the sperm clumps observed in collections 10-12, after the ampullae were unblocked. Ejaculatory failure can be multifactorial and identification of the cause is paramount to resolving the problem. Herein, uncontrolled orthopedic pain prevented frequent attempts at ejaculation which were required to relieve ampullae blockage. This case highlights a multidisciplinary approach to resolve a challenging case.

Keywords: Ejaculatory failure, sperm, pain management, horses

Fetotomy of a foal maldisposition (hurdling) due to a congenitally contracted hindlimb

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An 8yr-old Tennessee Walking Horse mare was presented to the Illinois Veterinary Teaching Hospital for dystocia lasting two hours. Obstetrical evaluation revealed an enlarged foal in cranial presentation, dorsal iliac position, with forelegs crossed over the head and neck ("foot-nape position"). Fetal malposture was corrected and obstetrical chains placed for traction in conjunction with uterine and abdominal contractions. The foal was successfully protruded from the vagina up to hip level, however, despite continuous traction, rotation, and additional lubrication, delivery was unsuccessful. Hip-lock or hindleg fetal malpostures were suspected. Examination revealed congenital contracture of the right hindlimb, placing the foal in a hurdled malposture. The mare had severe hindlimb paresis and ataxia, consistent with nerve compression. She was administered intravenous hypertonic saline, dextrose, and flunixin meglumine to enable transportation to the hospital. On arrival, the mare was induced under general anesthesia (GA) for fetotomy. A cut was made at the foal's femoral epiphysis to release its hips and left hindlimb, allowing the right hindlimb to be easily removed. Reproductive evaluation revealed major vaginal and vulvar bruises but no tears. The placenta passed shortly after oxytocin (20IU IV) administration. Postpartum care consisted of intravenous fluids for 24h, gentamicin (6.6 mg/kg IV), procaine penicillin G (22,000 IU/kg IM), metronidazole (10 mg/kg PO), flunixin meglumine (1.1 mg/kg IV), tetanus toxoid, thiamine (2g IM) and dexamethasone (50 mg IV). The mare had severe bilateral hindlimb paresis and took two hours to stand after GA. Uterine lavages were performed daily, and Quadritop ointment was applied to her cervix and vagina to aid healing. The mare was monitored for postpartum complications (metritis, necrotic vaginitis, and laminitis) and discharged from the hospital after 3d with instructions to administer oxytocin (20UI q 8hr) and trimethoprim sulfa (30 mg/kg PO q 12h). She was found to be healing properly at 7d recheck.

Keywords: Obstetrics, mares, postpartum, dystocia

Monozygotic twins from a mare following transfer of single embryo

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The occurrence of twin pregnancies in mares is an undesirable event. Fortunately, the equid has evolved to naturally self-reduce one of the pregnancies if they are positioned unilaterally. There are reports of increased rates of monozygotic twinning when assisted reproductive technologies (ART) are utilized in human medicine and a few reports in the veterinary literature have indicated this may also be a factor in mare ART.

A Quarter Horse embryo donor mare was managed for breeding and on day 7after ovulation a single embryo was recovered. At the time of embryo recovery the practitioner subjectively noted the recovered embryo was large for its embryonic age. The embryo was then washed and non-surgically transferred into a healthy, synchronized recipient mare. Pregnancy was diagnosed via ultrasound and viable twins were identified at 31 days gestation and again at 40 days gestation. The plan was made to reduce one of the twin pregnancies via cranio-cervical dislocation. At 54 days gestation a colpotomy was performed and the cranio-cervical region of the fetus was manually grasped and the cervical vertebrae were dislocated until the dorsal vertebral ligament was palpably ruptured.

The co-twin fetus remained viable on subsequent ultrasound evaluations performed at three and five months gestation. The pregnancy went to term and a healthy, normal sized foal was born along with a mummified fetus encased within its own fetal membranes. Genetic testing was performed on both the foal and mummified fetus and monozygocity was proven. This case demonstrates the need for continued ultrasonographic monitoring of early pregnancy in a recipient mare in spite of a single embryo being transferred. Complications from twin pregnancies can result in the loss of life of both the mare and the fetuses if allowed to go to term and a dysotica ensues.

Keywords: Mare, monozygotic, twins, embryo transfer, colpotomy

Can a foal survive ascending placentitis, uterine inertia, fetal maldisposition, neonatal encephalopathy, uroperitoneum and pharyngeal dysfunction with secondary aspiration pneumonia?

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A 23-year-old, multiparous, pregnant Quarter Horse mare (310d) was presented with purulent vulvar discharge and premature mammary gland development. Transrectal ultrasonography revealed placental edema and separation at the cervical star. The mare was treated orally for ascending placentitis (trimethoprim-sulfamethoxazole [TMS] 30mg/kg q12h; flunixin meglumine [FM] 1.1mg/kg q12h 7d, and altrenogest 0.088mg/kg q24h) until delivery. At 340d gestation, the mare had amber colored vaginal discharge without abdominal contractions. Obstetrical evaluation revealed chorioallantois rupture, dilated cervix, absence of uterine contraction consistent with primary uterine inertia, and a fetus in cranial presentation, dorsal iliac position, with bilateral carpal flexion and lateral deviation of the head and neck. Maldisposition was corrected and the foal delivered with assistance. Postpartum maternal care included correction of hypocalcemia and hypomagnesemia with IV lactated Ringer's solution (+150ml CMPK/10L), pain management FM (1.1mg/kg IV q12h), placentitis treatment TMS (30mg/kg PO q12h), oxytocin (10 units/IM q6h) and episioplasty for correction of abnormal vulvar conformation. The foal required extensive prolonged resuscitation and subsequent intensive care for neonatal encephalopathy, failure of passive transfer (plasma transfusion 2L) and presumed sepsis (cephalexin 30mg/kg PO q8h). On d5, uroperitoneum developed secondary to urachal tear and was surgically corrected. Appropriate mentation and suckle were present on d10, but dysphagia was observed within 24h. On d11, upper airway endoscopy revealed pharyngeal weakness and dysfunction. Thoracic imaging (CT, ultrasonography) and trans-tracheal aspiration results were consistent with aspiration pneumonia and acute respiratory distress syndrome. This resolved with antimicrobial treatment (cephalexin 30mg/kg PO q8h; metronidazole 15mg/kg PO q8h) and prednisolone sodium succinate (0.5mg/kg IV q12h then tapered). The filly was weaned and bucket-fed due to persistent pharyngeal dysfunction. The filly was discharged on d41 and is now a healthy yearling. This case highlights clinical problems associated with high-risk pregnancy, including intra/postpartum complications, resulted in a successful outcome.

Management of a cervical prolapse in a pregnant 1-year old Katahdin ewe

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A 1-year old 55 kg pregnant Kathadin ewe with a body condition score of 5/9, expected to lamb at any time was diagnosed by the ambulatory services with a grade 3 vaginal prolapse. Treatment included a sacro coccygeal epidural with 2% lidocaine followed by manual reduction of the prolapsed tissue. The cervix was determined to be 1-2 finger dilated. A Buhner suture was placed with umbilical tape to prevent recurrence of the prolapse. Antibiotics and anti-inflammatory were given and owner educated regarding the importance of prompt removal of the suture upon onset of labor.

Four days after initial examination she was presented to the teaching hospital in labor with recurrent grade 3 vaginal prolapse inducing a dystocia. Elective cesarian section was performed. Two large ram lambs (one alive and one dead) were delivered. Postoperative anti-inflammatory, antibiotics and prostaglandin F2α were given to the ewe.

The ewe and lamb were discharged from the hospital and was owner instructed to monitor for further signs of illness. Vaginal prolapse in sheep most often occurs in late gestation. A Buhner suture is a routine treatment for vaginal prolapse and works by replicating the vestibular constrictor muscles.[1] It is essential for the ewe to be closely monitored as impending parturition ensues, as a catastrophic outcome can occur for both the ewe and lambs if the suture is not untied prior to delivery resulting in trauma and/or death to animals. The most common etiologies include, short tail docking, large fetuses and overconditioning of the dam, as well as, genetic predisposition.[2] This case warranted extensive client education due to known high genetic merit associated with the flock.[3] Kathadin sheep are typically not tail docked, therefore further sire or genetic traits must be studied. Upon full recovery of this patient, culling was recommended to avoid potential genetic spread of this condition.[2]

Keywords: Ovine, prolapse, dystocia, cesarian, genetics

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A case of fescue toxicity in a broodmare

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An 11 year old pregnant mare was presented with prolonged gestation and aglactia. At 362 days of gestation, a transabdominal ultrasound was performed. Fetal heart rate and placental thickness were normal. The mare exhibited poor progression from 338 to 370 days gestation. The primary concern was prolonged gestation and lack of udder development, indicative of fescue toxicosis. Administration of domperidone (1.1mg/kg orally) began on 365 days gestation. On day 371 of gestation, the mare's mammary gland was engorged and waxing was present. She foaled that afternoon. The placenta prematurely separated and an assisted foaling was required.

After foaling, the mare incompletely expelled her placenta and retained the tip of the non-gravid horn. Treatment for the retained placenta included uterine lavage, administration of oxytocin, and flunixin meglumine. The retained portion of the placenta was retrieved within three hours. Grossly, the placenta was abnormally thickened. Colostrum quality was a concern because of the poor mammary development prior to foaling. Colostrum quality was tested via Brix refractometer at 18%, or "fair" quality. The foal's IgG level was measured 18 hours after foaling at 673.4 mg/Dl, indicating partial failure of passive transfer. Plasma was administered to the foal and domperidone was continued in the mare for three days to assist milk production.

Fescue toxicity occurs when mares consume endophyte-infested fescue during the last third of gestation. Endophyte infested fescue contains a deferential toxin, ergovaline, that function as a dopamine D2 agonist which decreases prolactin.[1] Clinical signs include prolonged gestation length, aglactica, thickened placenta, and weak foals.[2] Treatment with a dopamine antagonist, domperidone, has been demonstrated to be effective in decreasing gestation length and increasing milk production.[3] This case is important to the study of theriogeneology because it documents the importance of proper diagnosis and management of broodmares on fescue during the end of gestation.

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Uterine torsion and concurrent uterine rupture in a Brown Swiss cow

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A twelve year old Brown Swiss cow was presented to the Veterinary Teaching Hospital with a history of being moderately depressed for the past twenty-four hours. Upon rectal palpation, a calf was evident along with a tight band on the left side of the pelvis consistent with a uterine torsion. A vaginal examination was performed and a decreased lumen was apparent in the cranial vagina along with distortion of the vaginal wall further supporting a diagnosis of a uterine torsion. To correct the torsion the cow was rolled with a plank holding the uterus in a fixed position. At this point the torsion was corrected but the cervix was not fully dilated. The decision was then made to perform a left flank cesarean section. Ampicillin, flunixin meglumine, and intravenous Ringer's solution were given pre-operatively. During surgery, it was noted that the uterus had ruptured and there were several large blood clots present in the abdomen along with multiple tears in the uterus. A dead 120 lb. heifer calf was extracted and the tears in the uterus were repaired with 3 chromic gut. The caudal portion of the tears was repaired using a simple continuous pattern while the more cranial portion was repaired using a Lembert suture pattern. Treatment with ampicillin was continued postoperatively and the cow was discharged three days later with a fair prognosis. The main concern at the time of discharge was the development of peritonitis. The reproductive potential was expected to be reduced due to the severity of the uterine rupture. However, a year later she was successfully rebred via artificial insemination. This case is a rare example of a cow able to return to reproductive function after severe uterine trauma.

Keywords: Uterine torsion and rupture, cow

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