



Clinical Theriogenology

Official Journal of
The Society for Theriogenology
The American College of Theriogenologists

Clinical Theriogenology

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Clinical Theriogenology

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Clinical Theriogenology

Official Journal of Society for Theriogenology and American College of Theriogenologists

Mission Statement

Purpose of Clinical Theriogenology is to publish in a timely manner peer-reviewed information relevant to clinical practice in theriogenology for veterinary practitioners, academic clinicians, and veterinary students. Society for Theriogenology publishes its proceedings of Annual Conference and Symposia in Clinical Theriogenology.

Scope of the Journal

Clinical Theriogenology is broad in scope. Following types of manuscript are published in addition to news from the Society for Theriogenology and the American College of Theriogenologists.

- Research report
- Review report
- Case report
- Technique report

Publication Schedule

Regular issues will be published quarterly.

Manuscript Preparation

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

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- 1" margins at the top, bottom, and sides of each page
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- All lines of manuscript should be numbered consecutively
- Title: Should be self-explanatory. Bold font, centered, only first word and proper nouns upper case, remainder lower case, e.g.

Hydroallantois and prepubic tendon rupture in a Standardbred mare

- Authors: Use only first name and last name; separated by comma; departmental or institutional affiliation denoted by superscript letter (superscript numbers are used to cite references); superscript placed after punctuation; centered below title; do not include degrees or specialty board certification, e.g.

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- Text: Leave a space below **Keywords** and start the text and divide it into traditional sections of a manuscript, namely, introduction, headings, subheadings, and conclusion. Indent the first line of each paragraph by 0.5 inches; single spaced; do not leave extra space between paragraphs. Leave a space before and after headings and subheadings.
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Hydroallantois in mares is a rare and life-threatening condition,^{1,2} which may lead to abdominal wall disease.^{2,3}

If 3 or more references are cited, use a hyphen between the first and last one, do not list them all, e.g.

Blood pressure rises when linea alba is incised and as ovaries are manipulated in healthy bitches undergoing OHE.¹⁵⁻¹⁷

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Journals

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List only first 3 authors, if more than 3, then use et al. Use only last name of authors first, then initials, no periods after initials or spaces between initials, comma between authors' initials, colon (:) after last author initials or after et al, e.g.

Clark SG:

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Year of journal publication is followed by a semicolon (;), no space after year or after semicolon.

Volume Number

Volume number of the journal follows the semicolon, no space and is followed by a colon, issue numbers, months, etc. are not included.

Page Numbers

The inclusive page numbers are listed; if only the first page is listed, it gives the appearance that the work cited is an abstract. Use the shorter hyphen (-) between the first and last pages. A period follows the last page number (no abbreviation of last page), e.g.

Journal article (single author)

Odde KG: A review of synchronization of estrus in postpartum cattle. J Anim Sci 1990;68:817-830.

Journal article (more than 3 authors)

Martinez MF, Adams GP, Kastelic JP, et al: Induction of follicular wave emergence for estrus synchronization and artificial insemination in heifers. Theriogenology 2000;54:757-769.

Book (single editor)

Krekler N, Hollinshead F: Pyometra. In: Monnet E: editor. Small Animal Soft Tissue Surgery. 1st edition, Hoboken; Wiley-Blackwell: 2013. p. 625-634.

Book (more than 3 editors)

McKinnon AO, McCue PM: Induction of ovulation. In: McKinnon AO, Squires EL, Vaala WE, et al: editors. Equine Reproduction. 2nd edition, Ames; Wiley-Blackwell: 2011. p. 1858-1869.

Proceedings

Kenny RM, Bergman RV, Cooper WL, et al: Minimal contamination techniques for breeding mares: techniques and preliminary findings. Proc Annu Conv Am Assoc Equine Pract 1975; p. 327-336.

Outline for Case Report

Title of Case

Authors of case: Only use first and last names of each author and institutional affiliation

Do not list academic degrees or specialty board certification

Abstract: Up to 150 words summarizing case presentation and outcome

Background: Why is this case important? Do not describe the case here but provide information from literature and ‘make a case for your case’

Please go over published cases in Clinical Theriogenology

Case Presentation: Presenting features, pertinent medical history, and herd history, if applicable

Differential Diagnosis, if relevant

Treatment

Outcome

Discussion: Include a brief review of similar published cases; how many other similar cases reported?

Learning points: Three to 5 bullet points

References: Similar to research report (refer manuscript preparation)

Figure/photo captions, if any

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Dear theriogenology friends,

Hopefully this conference issue will be very useful to follow along for those who will be participating in the ‘virtual platform’ of the conference. To others who are not attending virtually, there is wealth of information on these pages. Conference leaders invited scholars working in various aspects of theriogenology to share their expertise and truly we are the beneficiaries of their willingness to contribute amidst their busy schedule for the benefit of many. We are also thankful for the session chairs for their time and efforts to plan and deliver this meeting.

Theriogenology will continue to make progress in all aspects of its mission; quality abstracts submitted to the committee is only 1 proof of it. I thank the abstract committee for their dedication and perseverance. Research and case abstracts from undergraduate students are a ‘breath of fresh air’ and surely theriogenology will flourish because of strong mentorship, critical thinking, and intellectual creativity that exist in our discipline. It was so encouraging to receive a following note (in quotes) from a student in response to my request to approve an edited abstract ‘I approve of the edits. Thank you for your hard work on this, as I’m sure reformatting all the abstracts for consistency is no easy feat. It is greatly appreciated.’ All students who participated and Dr. James Alexander, organizer of student abstracts, deserve our appreciation.

Normally, the journal does not publish presentation of a ‘Theriogenologists of the Year’ awardee. We did it this year, since I believe it will motivate many as they read the ‘theriogenology journey’ of our colleague, Professor Claire Card. Theriogenology, will continue to shine in academia and in practice for it has the best and the brightest talent and it is the ‘back-bone of veterinary medicine’. Please be sure to read Professor Ahmed Tibary’s surgery paper; our discipline will have a tremendous impact in academia, if we continue to foster and grow reproductive surgery in our programs in collaboration with surgeons.

I will be remiss, if I do not recognize the efforts of our ‘point person’ for conference papers and abstracts in our management office, Ms. Tara Beasley. Tara, thank you for your patience and persistence. I also sincerely appreciate Professor John Kastelic’s relentless effort to bring consistency to copyediting.

Regards,

Augustine

The 2020 Bartlett address

Terry Blanchard

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I wish to thank the American College of Theriogenologists, Society for Theriogenology, and Theriogenology Foundation for bestowing this award on me. As I remember, this award was originated to honor our forefathers responsible for creating our specialty college. The members that founded the Rocky Mountain Society for the Study of Breeding Soundness comprised most of this group. Their vision and perseverance are legendary to our members. They set the bar high. As such, I can't help but feel that my professional career doesn't approach this standard.

My interest in Theriogenology was peaked when I was in the US Peace Corps in the Philippines. I was recruited to help develop artificial breeding strategies in the native water buffalo (carabao). Funds available for this project were delayed for some time, and I was asked to teach animal science and pre-veterinary college students. I discovered that I really enjoyed teaching.

I am reminded that no one has a meaningful career uninfluenced by others. I was fortunate to be selected for a large animal reproduction residency with Bob Kenney at New Bolton Center. While at New Bolton Center, I was also mentored by Wendell Cooper, John Hurtgen, and Marolo Garcia. These amazing mentors were the beginning of many that suffered through my presence and instructed me patiently and tirelessly. Other mentors that greatly influenced my career include my graduate advisor Ron Elmore, Bob Youngquist, and Clarence Bierschwal at the University of Missouri. On my return to New Bolton Center, I was fortunate to work with Dickson Varner, Katrin Hinrichs, and Stan Brown. While earlier in their academic careers, I believe I learned more from them than they from me.

I later joined Dickson at Texas A&M University. This was the beginning of a long and storied career shared with Dickson. He has always been the visionary that brought me along for an amazing ride. I also would not have enjoyed a meaningful career without the enduring support of Ron Elmore, Ron Martens, and Bill Moyer at Texas A&M University. Finally, veterinary students and residents I taught that have become lifelong friends as well as colleagues include Jim Moorehead, Charlie Love, Steve Brinsko, and Margo Macpherson and numerous others.

Again, I reiterate my gratitude to the American College of Theriogenologists, Society for Theriogenology, and Theriogenology Foundation for this award.

Theriogenologist of the Year in the SARS-CoV-2 pandemic

Claire Card

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To be nominated and chosen as the Theriogenologist of the Year (TOY) is a great honor and I am deeply humbled by this award. I am also deeply saddened that my colorful Big Apple New Yorker friend Peter Brunelli of Universal Ultrasound, who died of COVID19, and who has sponsored this award, is not here today for me to thank. Peter used to chide me about being “the theriogenologist who practices what she preaches,” reminding everyone within ear shot that I had 4 children and 2 stepchildren! Peter was an incredible supporter of the American College of Theriogenologists and the Society for Theriogenology, and I will miss him dearly.

This has been quite a year to reflect upon in the backdrop of the Coronavirus Severe Acute Respiratory Syndrome coronavirus-2 (SARS-CoV-2) pandemic. A word cloud of the Pandemic would be filled with: Zoonosis. Pangolins. Bats. Cats. Ferrets. Mink. SARS. MERS. Emerging disease. Public health emergency. Air born. Respiratory droplet spread. Pneumonia. Ventilator. Borders closed. Social distancing. Quarantine. N95. Facemasks. PPE. Community spread. Shelter in place. Vulnerable populations. Doubling rates. Flattening the curve. Fomites. Contact tracing. Self-quarantine. Supershedder. Reproduction number R0, Case Fatality Rate. Crisis.

Reviewing the past awardees of TOYs is impactful. My thoughts included: what an incredible list, how fortunate I am to have known these people, how our profession has been advanced by their scientific discoveries, how we need to encourage the nomination of more women, and how the heck did my name get on the list? This is my opportunity to thank those who illuminated my path. I want to acknowledge my formative years at Cornell University with mentors: Ron Gorewit, Bruce Currie, Peter Nathanielsz, Mary Smith, Joanne Fortune, my PhD supervisor Ron Butler, and my committee members Don Schlafer, and Bill Hansel; members of the Theriogenology section: Drs. Bob Hillman, Rob Gilbert, Barry Ball, Tom Little, Peter Daels, and Vicki Myers Wallen; and my fellow grad students Lisa Freeman, George Haluska, Rick Canfield, Charlie Elrod, and Susan Huyler. Your help and support were invaluable.

I owe a great deal to the dedication and iron work ethics of the students I supervised at the Western College of Veterinary Medicine’s combined residency and graduate program, including Drs: Shawn Haas, Stephen Manning, Theresa Burns, Natalie Bragg, Farshad Maloufi, Alejandro Rey, Alexandra Rauch, Farhad Ghasemi, Tal Raz, Sarah Eaton, Dawn Nairne, Mariana Diel de Amorim, and Maria Lopez Rodriguez, and the other graduate students I had the opportunity to mentor. You have all been an inspiration, and watching you become talented and sought-after clinicians and scientists has been a highlight for me. Similarly, thanks go out to my many collaborators over the years: Drs. Ed Squires, Jason Bruemmer, Irwin Liu, Don Thompson, Claudia Klein, Elemir Simko, Bernard Laarveld, Nadia Cymbaluk, Tasha Epp, Gregory Starrak, and Robert Foster along with the sea of undergraduate students, now veterinarians: Drs. Mark Corrigan, Jodyne Green, Sylvia Carly, Barb Hunter, Allister Gray, Ilse Dedden, Ellie Ripley, Jasmine Paulson, Patrick Roberts, Lyndsay Rogers, Megan Jurasek, Kayla Nielsen, Brad McKell, Mikayla Swirski, Brandi Bakken and Ashlyn Ketterer. Thanks to Tammy Kimmel and veterinary technicians: Mikhaela Thasher, and Rebecca Johnston, along with the Mason family’s Hilltopper Clydesdales, Ron Schreiner of D and R Ranch, and Jack and Linda Iveson of Jaclyn Quarter horses. A big thank you to all of our clients and referring veterinarians who have shown great trust in us over the years. Lastly all of the members of the ACT, SFT, and TF family who are my friends and whom I have had the pleasure of knowing and serving with over many years. You have made our veterinary speciality Theriogenology vibrant, relevant and progressive.

The award is often given to acknowledge significant recent research contributions. Our research contributions from Dr. Diel de Amorim's PhD work have centered on the role of oxytocin (Oxt) and oxytocinase,^a an enzyme that metabolizes oxytocin, in the maternal recognition of pregnancy (MRP) in mares, most of that is under review. If I had to describe the foundation for our research work, it was built on the work of those who came before. Ground breaking work was done in the early days by Dan Sharp on prostaglandin,^{1,2} Pat Sertich on embryonic and endometrial prostaglandin secretion,³ Elaine Watson^{4,5} on endometrial and luteal oxytocin, Gordon Woods and Jim Weber on embryonic PGE section,⁶ Dirk Vanderwall on PGE and the effects of oxytocin on the corpus luteum,⁷ Claudia Klein and Mats Troedsson⁸ on molecular aspects of MRP, Bob Douglas and Ollie Ginther on prostaglandin,⁹ Christine Aurich and Sven Budik on interferons and oxytocin receptors,¹⁰ Tom Stout and Twink Allen on embryo migration and the endometrial luteolytic pathway,^{11,12} along with many others. We have expended considerable effort to understand some of the complexities of MRP in the mare. We confirmed that oxytocinase was present in many equine tissues, including luteal, uterine and trophoblast tissue. Oxtocin is produced as a preprohormone bound to neurophysin -1 and is then processed into the active hormone Oxt. We showed that whereas luteal tissue expresses Oxt, there is little hormone produced, making luteal origin oxytocin a possible intraluteal paracrine regulator, but an unlikely regulator of endometrial prostaglandin secretion. There is more work to be done to understand the role of Oxt gene expression and posttranslational processing of the oxytocin/neurophysin prohormone Oxt protein in MRP.

We have also another research focus from the thesis work of Dr. Maria Lopez Rodriguez, that is related to equine thyroid function and fetal development. In the early North American veterinary literature, the birth of contracted legged abnormal foals was identified.¹³ Gross and histologic thyroid abnormalities were reported and associated with low concentrations of iodine in the prairie soil.^{14,15} Outbreaks of disease¹⁶ and foals with severe musculoskeletal abnormalities including: dysmaturity, hypothermia, severe limb contracture, rupture of the common digital extensor tendons, mandibular prognathism, abnormal umbilicus, thyroid metaplasia and carpal/tarsal boney dysgenesis were identified¹⁷. This collection of abnormalities was termed congenital hypothyroidism dysmaturity syndrome (CHDS).¹⁸ Equine fetal studies including thyroidectomy¹⁹ proved that fetal thyroid hormone was actively secreted in the last third of gestation²⁰. Equine fetal thyroidectomy showed the critical importance of the thyroid in equine fetal development, and recreated many of the clinical signs of CHD.¹⁹ Research work by others highlighted the epigenetic effects of thyroid hormone on fetal development.^{21,22} Investigators identified that no trace mineral supplementation¹⁷ and exposure to mustard plants^{23,24} were a risk factor for CHDS. Mustard plants contain compounds called glucosinolates that are metabolized into goitrogens and alter thyroid function²⁵. Our studies showed that feeding glucosinolates combined with a low iodine diet resulted in reduced serum iodine concentrations similar to the effects on other species^{2,5-27}. We demonstrated that broodmares often have insufficient concentrations of iodine, in spite of access to salt blocks and mineral supplements.²⁸ Depending on the voluntary consumption of salt blocks or mineral products by broodmares to meet their iodine and other trace mineral needs may lead to insufficiency. We also reported that equine mammary tissue concentrates iodine and is a source of iodine for the foal.²⁸ Equine neonates have very high concentrations of serum iodine, total thyroxine and total triiodothyronine, all of that decrease rapidly over the first 10 days of life and play a key role in neonatal adaptation. However, questions still remain and further studies are needed with known and confirmed intakes of iodine to confirm the appropriate reference concentrations.

What have my years in research taught me? What would that word cloud look like? Team. Determination. Hard work. Repeat. Redo. Redesign. Focus. Literature. Hypothesis. Gene. Protein. ELISA. Western Blot. Mass Spec. Immunohistochemistry. Resilience. My family, my husband Peter Barnacle, daughters Kirstin, Ursula, Johnna and son Ryan, step-children Shawn, Tricia and grandchildren

^a Oxytocinase is also called leucyl-cystinyl-aminopeptidase (LNPEP), insulin responsive aminopeptidase (IRAP) and placenta leucine aminopeptidase (PLAP)

Owen, Jake, Nicholas, and Samantha, along with my good friends helped me survive. Much of my success is due to their support. It was not easy being a female large animal veterinarian and I hope that my efforts have made it easier for those who follow.

Key messages I wish to give to others is that the amount of funding for equine and small animal research remains woefully inadequate and needs to improve. This will rely on our profession's advocacy for changes in government policy and continued private and industry support for foundations such as TF and other granting agencies. My partnership with rural women farmers in Uganda has taught me that the true measure of one's life is not about the things money can buy, but in how much we give to others.

As investigators, I encourage everyone to continue to ask questions, and to keep checking your sources. Additionally, to members of the public, respect science, it is the pathway out of ignorance, out of the pandemic and out of the pernicious challenges that lie ahead, including rapid species extinction and climate breakdown.

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The future is coming! New technologies being applied to reproduction in animals and humans

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Abstract

Advances in genome sequencing, gene editing and computational analyses have brought the genetic future rushing toward us. Gene editing is currently being used extensively in research, to explore function of genes via over- and under-expression, and to produce animal disease models and animals useful for production of medically important components. Through computational analysis of nucleotide sequence versus performance, disease or other morphological characteristics, tens of thousands of genetic variants associated with a large number of traits have been identified in many species, and selection based on genetic merit, even in preimplantation embryos, is ongoing. Advances are also being made in understanding and manipulation of primordial germ cell and oocyte development, such that functional oocytes have been produced from somatic cells; and in extended in vitro culture of embryos past the implantation stage. Combined with work on development of an artificial womb, the potential to achieve extra corporeal pregnancy seems plausible. Many of these advances appear to warrant veterinary, breed registration or ethical oversight, so it is important that veterinarians be aware of new achievements in these areas.

Keywords: Oocyte, embryo, stem cells, gene editing, uterus

Gene editing

Claudia Klein at the 2019 Society for Theriogenology Annual Conference presented an in-depth review of methods for gene editing and provided useful references regarding these techniques.¹ Gene editing methods have come to the forefront; most useful are **TALENs** (Transcription Activator-like Effector Nucleases) and **CRISPR** (Clustered Regularly Interspaced Short Palindromic Repeats).

TALENs are proteins. They recognize specific sections of DNA via similar mechanisms to those that transcription factors use: the protein structure of the TALEN binds only to a specific nucleotide sequence. TALENs are linked to effector proteins.

CRISPERs are RNA nucleotide sequences. One section of the CRISPER is designed to be complementary to the target DNA nucleotide sequence and so recognizes and binds to that specific section of DNA; the second section of the CRISPR molecule is associated with an effector protein.

Both TALENs and CRISPRs work via the **effector proteins** associated with them. For gene editing, the linked effector protein is an endonuclease (an enzyme that cuts DNA); the specific endonuclease CAS9 (CRISPR-Associated protein 9) is typically linked to CRISPR. When a TALEN or CRISPR is bound to the recognized nucleotide sequence, the endonuclease will cut the DNA at a specific nucleotide, adjacent to the location of the TALEN or CRISPR. Since the first use of guided endonucleases to induce site-specific DNA breaks,²⁻⁴ many other kinds of effector proteins have been linked to these targeting molecules: proteins that cause a gene to “turn on” and start transcription; proteins that inhibit transcription and even proteins that deliver epigenetic markers (reviewed⁵). Linkage of these proteins to targeting molecules that recognize specific nucleotide sequences allows the investigator to modify the DNA sequence, gene activity, or epigenetic profile of targeted areas of genome.

The most straightforward use of gene editing tools is to create a mutation that causes a gene to be inactive. For this, editing tools are designed to make a double-stranded cut in DNA, which the cell finds difficult to repair while maintaining fidelity. Double-stranded repair thus is typically associated with changes to the nucleotide sequence resulting in reading frame shifts in DNA, and thus in a mutation that can completely inactivate gene.

It is also possible to change the sequence of a gene in a directed way, by making a break and providing the cell with multiple copies of a template, i.e. pieces of DNA with the desired nucleotide sequence. The cell uses the template to direct repair of the cut section, resulting in the desired genome

sequence in some cells. Remarkably, it is not necessary in all cases to provide a template; if the homologous chromosome has a different sequence for the targeted gene (which thus is not affected by the editing tool), mammalian cells can use the homologous chromosome as a template to fix a double strand break.⁶ In this way, cells that are heterozygous for an undesired mutation can “fix” the gene sequence in mutated gene, without introduction of foreign DNA.

Gene editing tools can be used to produce animals with a desired genetic modification in 2 main ways: 1) by editing somatic cells, selecting a cell with the desired modification, and performing somatic cell nuclear transfer (SCNT); or 2) by injecting the editing tool into a zygote or early embryo, thereby directly editing the genome of the zygote/embryo and thus all resulting embryonic cells. A third area of intense interest is using these tools to correct disease-related genetic mutations in living animals (gene therapy).

Gene editing has been used in almost all domestic species, including horses (embryos with knockout for myostatin, produced by SCNT)⁷ and dogs (gene therapy to correct the muscular dystrophy gene;⁸ production of myostatin-knockout dogs via zygote injection).⁹ It has also been applied to a wide variety of nondomestic animals, including macaques, ferrets, koi, zebrafish, squid, shrimp beetles, butterflies, and bees, to study genetic factors related to their behavior and health.^{10,11} The ethics of gene editing to “fix” mutated genes in human embryos is currently being debated.

Problems with POLLED gene-edited calves

Stories involving gene editing have recently been in the news. An American company, Recombinetics, published a report in 2016, announcing production of 2 crossbred dairy bulls produced via SCNT with somatic cells edited with TALENs, that were consequently homozygous for the POLLED allele.¹² The company termed this method “precision breeding” (<http://recombinetics.com/naturally-hornless-cattle/>). They proposed that this would be a good model for introduction of gene-edited livestock into food production, as progeny of these animals would not require debudding to remove horns, a procedure with animal welfare concerns. However, genome sequencing of the gene-edited bulls’ progeny (University of California, Davis) revealed a bacterial antibiotic-resistance gene in some calves’ DNA (apparently, transferred to cells during the editing process) that was not detected in the original testing of the bulls.¹³ These findings represent a dramatic setback to possibility of acceptance of gene-edited livestock for enhancement of production, both because of presence of “off-target” changes in the animals’ DNA and because the problem was not revealed during initial genetic testing.

Birth of gene-edited human babies

In a second event regarding gene editing, a scientist in China, He Jiankui, announced via a video released on YouTube in November, 2018, the birth of human twins which he claimed had undergone gene editing as zygotes. The editing was to induce a genetic modification, $\Delta 32$ mutation in the CCR5 gene, which when naturally occurring confers resistance to entry of a common variant of HIV virus. This human experimentation violated Chinese regulations banning genome editing on human embryos, as well as basic scientific and ethical guidelines (<https://www.nytimes.com/2018/12/05/health/crispr-gene-editing-embryos.html>) and was condemned by scientists in China and abroad.¹⁴ The Scientific Ethics Committee of Academic Divisions of the Chinese Academy of Sciences stated that “the theory is not reliable, the technology is deficient, the risks are uncontrollable, and ethics and regulations prohibit the action.”¹⁴ The gene-edited embryos were apparently biopsied to confirm presence of mutation, and then transferred, resulting in birth of twin girls. However, further evaluation of the process used revealed that the editing process was not precisely directed, and that both children have a mosaic of different mutations of the targeted gene, which may or may not confer resistance to HIV. He Jiankui was fired by his university, and in December 2019 was sentenced to 3 years in prison; the court found that this researcher and his collaborators forged ethical approvals and did not inform the physicians transferring the embryos of their origin.

Use of gene-edited mice and time-lapse photography to study embryo development

The study of embryo development has progressed markedly with the use of time-lapse, within-incubator photography, currently utilized extensively in human assisted reproductive technology (ART; reviewed¹⁵) and recently applied to clinical equine IVP embryos.¹⁶⁻²⁰ Work in mouse embryos has gone a step further: using the precise and efficient new gene editing tools, mice have been produced in which key cytoplasmic components were labeled with fluorescent markers. Combining these markers with fluorescent molecular labels and using time-lapse fluorescent microscopy, it is possible to visualize changes in cytoskeletal, nuclear and cell-lineage specific components during embryo development.²¹ Using information gained in this manner, scientists are beginning to outline morphogenetic mechanisms involved in early embryo and blastocyst development.²²

Embryo biopsy, testing and selection

Cattle

The availability of immense amounts of production data on bulls, cows, and their progeny has allowed identification of hundreds of thousands of genetic markers (single polymorphic nucleotides, SNPs); tens of thousands of which have been associated with desirable traits, both in beef and dairy cattle. Microarrays for rapid detection of production-related SNPs are marketed commercially and have been used since the mid 2000s²³ to select calves for genetic merit at birth, resulting in rapid and meaningful gains in productivity.²⁴ These genetic selection methods are now being applied to embryos, via cells derived from embryo biopsy, to allow selection of embryos before transfer.²⁵ The ability to select embryos with high genetic merit, combined with the ability to recover competent oocytes from gonadotropin-treated heifer calves as young as 2 months of age for in vitro embryo production,²⁶ are expected to result in greatly accelerated genetic gain through decreasing generation time.

Horses

Methods for effective biopsy and genetic analysis of both in vitro-produced and in vivo-derived equine embryos have been developed.^{27,28} Currently, this procedure is used clinically, mainly for testing embryos for presence of disease-related mutations²⁹ or for fetal sex.³⁰ Because horses lack the immense database found in cattle, information on genetic markers for performance in horses is more difficult to generate; however, an increasing amount of information is available in this area. In a 2010 report, variants of myostatin gene, apparent as SNPs, were found to be strongly associated with success of horses at different racing distances.³¹ Subsequently, equine SNPs were identified that are associated with “gaitedness”, e.g. found in horses that deviate from standard two-beat trot and instead perform running walk, rack, singlefoot, etc.;³² with size, e.g. miniature, pony, horse, draft horse;³³ breed type;³⁴ and with selection for speed in Australian thoroughbreds, via SNPs in a chromosome location associated with neuromuscular junction signaling.³⁵ Because effective methods for embryo biopsy are being developed in horses, the potential for preimplantation genetic selection in horses, as done in cattle, is growing.

Humans

Preimplantation genetic testing (PGT) has been performed on in vitro-produced embryos since the 1990's in couples known to carry disease-related mutations.³⁶ The term PGT refers to evaluating genetics of cells recovered on biopsy of embryo (embryonic blastomere of an early-cleavage embryo -- no longer used because of deleterious effects on embryo -- or trophoblast of a blastocyst). PGT can be divided into several types of assessments. Testing for a specific allele, typically 1 carrying a disease-related mutation (recently re-termed PGT-M; M for monogenic) was developed first. Subsequently, methods to evaluate the entire genome of cells obtained by biopsy were introduced, to determine aneuploidy (duplication or deletion of chromosome segments or entire chromosomes). Such genome-wide screening is currently termed PGT-A (A for aneuploidies). This screening was initially done by comparative genomic hybridization and is now done via genome sequencing. The jury is still out as to whether employing PGT-A to screen embryos before transfer actually increases the chances of live birth,

as there can be aneuploidies in the trophoblast that are not reflected in the embryo proper and the time needed for results to be available precludes fresh transfer of embryos.³⁷

The ability to evaluate the entire genome of embryos, however, has led to the possibility of selecting embryos for desired traits. Gender selection of human embryos (without a medical reason such as to avoid an X-linked disease) is illegal in some countries, but is legal, and offered, in other countries, including the US. Human ART labs are already offering selection for eye color and possibly other characteristics, and methods to select other traits, including intelligence (selection against genes known to be associated with a low IQ), have been developed and companies plan to market them (<https://genomicprediction.com/>). Human ART centers may soon be calculating a polygenic “risk score” for each embryo, based on known genetic markers of disease susceptibility, to allow selection of embryos likely to produce the healthiest individuals.

Recent advances with application to species conservation

Major barriers for SCNT use in conservation of endangered species are: 1) availability of oocytes from appropriate species, i.e. oocytes having species-specific mitochondria and cytoplasm compatibility with species’ genome, to use as host oocytes for SCNT; and 2) availability of a suitable recipient female – that is, a female that is fertile, cyclically synchronous with the embryo, capable of gestating the transferred embryo and in which embryo transfer can be performed (for example, there is no established method to transfer embryos to the rhinoceros uterus). However, several recent advances have potential for application in this area:

Production of functional oocytes from somatic cells

This possibility in mice was reported in 2012.³⁸ Somatic cells were first induced to be pluripotent by inserting genes coding for pluripotency factors, then these induced pluripotent stem cells (iPSCs) were caused to differentiate to primordial germ cell-like cells (PGCs). These were aggregated with embryonic ovarian cells and transplanted to the ovarian bursa of recipient females. The somatic-origin cells differentiated to oocytes and were recovered from the recipient ovary at the germinal vesicle stage and matured in vitro, fertilized in vitro and yielded live young.

In 2019, production of oocytes from somatic cells (granulosa cells) **without transgenesis** (using only chemical stimulation), using a similar series of procedures, was reported.³⁹ This is a huge leap forward toward eventual use of somatic cells for derivation of species-specific oocytes, even from totally extinct species. Many zoological parks have tissue or fibroblast samples from numerous species stored in liquid nitrogen; these somatic cells could be used for production of species-specific oocytes. Lack of embryonic ovarian cells from desired species to use for aggregating with somatic-origin PGCs should not be a barrier, since aggregation of oocytes, recovered from primordial follicles, with ovarian cells of other species is compatible with production of functional oocytes.⁴⁰

The prospect of deriving oocytes from somatic cells in this manner has great application in assisted reproduction to aid conservation of endangered species. Mitochondrial-identical oocytes could be used as host oocytes for somatic cell nuclear transfer, to produce clones using stored tissue samples. Even more exciting is prospect of such oocytes could be fertilized using stored sperm from males selected to produce offspring with greatest genetic diversity possible. In this manner, the population genetic diversity could be maximized and the genetics of animals for which only sperm is available could be reintroduced into the population.

Production of embryo-like structures directly from stem cells

The term “totipotent” is used to refer either to a cell capable of developing into a complete organism (e.g. a fertilized oocyte), or to a cell that can differentiate into any cell type of an organism. However, it was felt that these are 2 different issues.⁴¹ An oocyte can develop into entire organism after fertilization or nuclear transfer, whereas an embryonic stem cell (capable of contributing to any tissue of organism) cannot generate and organize entire organism. The term “plenipotent” has been proposed for this second, less capable cell.⁴¹

However, recent work has begun to blur these lines. Production of embryo-like structures from aggregated human stem cells is possible.⁴² Through a gel-based 3-D environment created by a microfluidic device, stem cells were induced to recapitulate early aspects of epiblast and amnion development, including cell line differentiation, lumen formation, polarity in the embryonic sac, specification of primordial germ cells and, notably, cells with primitive streak markers.

Derivation of induced pluripotent stem cells from somatic cells has been reported in several endangered species, including mandrill and northern white rhinoceros.⁴³ Thus, in the future, embryos might be derived directly from pluripotent stem cells. This opens the door to use of stored somatic cells to produce mitochondrial-identical clones of endangered and even extinct species without need for oocytes, or for going through the laborious steps of deriving primordial germ cells and then oogonia from stem cells.

Culturing embryos in vitro past blastocyst stage

Monkey embryos have been cultured in vitro for up to 20 days.⁴⁴ Embryos underwent differentiation to developmentally-appropriate cell lines and stages, including embryonic disc formation, amniotic and yolk sac cavitation, and differentiation of primordial germ cell-like cells. Human embryos have also been cultured in vitro past 7 days, with cell lineage differentiation, but culture was terminated at 14 days for ethical reasons.^{45,46} The ability to culture embryos in vitro to the point of differentiation and cell lineage specification is both unsettling, in terms of producing fetuses “in a test tube” and promising, in terms of being able to propagate embryos from endangered species without the need for a suitable recipient female. It appears that research will continue to push the ability to support embryonic development in vitro further and further into pregnancy.

Artificial womb

An in vitro method to support development of lambs during last 4 weeks of pregnancy has been developed.⁴⁷ Lambs were removed from the uterus and placed in a sterile polyethylene bag that was cannulated to allow continuous fluid exchange in an artificial “amnion,” and an oxygenating circuit was connected to the fetal umbilical cord. Extensive work was performed to optimize pressures, circuit flows, oxygenation and other mechanical aspects to reproduce the environment of the gravid uterus and support normal development of the lamb. With the optimized system, lambs at a stage equivalent to extreme prematurity in human infants survived for 4 weeks, had stable hemodynamics and normal growth, including lung development and brain maturation. Lambs were removed, ventilated and then euthanized to assess tissue development.

The potential application of this technique in ART for endangered species is immense. As in vitro early embryo culture methods continue to progress further toward supporting embryo development through the placental and fetal stages, perhaps these methods for supporting the fetus after organogenesis and umbilical cord formation will be applicable earlier and earlier in pregnancy, with the eventual possibility of supporting the entire pregnancy of an embryo/fetus without a species-compatible recipient female.

Conclusion

In many ways, science is continuing to explore techniques proposed for decades by science-fiction writers in an imagined future. Embryo production in vitro is commonplace; genetic selection is being used widely in cattle and is also utilized in human embryos, currently largely to avoid transferring embryos with devastating genetic diseases. Gene editing is being performed for research but has much interest from commercial sector, including human ART. It is difficult to predict future applications of these approaches; the ethical lines that society draws in these areas appear to move, as past innovative technology becomes commonplace and accepted.

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Seeking stem cell efficacy – immunogenicity matters!

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Abstract

Despite hundreds of publications and registered clinical trials, definitive evidence of mesenchymal stem cell (MSC) efficacy does not exist. The notion that mesenchymal stromal cells (MSCs) are immune privileged is changing: MSCs constitutively express MHCI, can be induced to express MHCII and allo-immunization against allogeneic MSCs has been documented. Despite the clinical safety of allo-MSCs, which has been shown exhaustively without evidence for acute rejection reactions or local tissue inflammation, immune recognition and rejection occurs with a reduction in MSC efficacy, we have demonstrated localized tissue inflammation secondary to immune recognition of allo-MSCs. We have also demonstrated localized tissue inflammation secondary to immune recognition of fetal bovine serum contaminants to MSCs. This localized tissue inflammation supports the clinical relevance of allo-recognition of MHC mismatched MSCs and FBS contaminated MSCs, despite the lack of clinically apparent adverse reaction. These findings may explain why definitive proof of MSC efficacy has not been achieved.

Keywords: Equine, bone marrow, mesenchymal, stem, stromal, regenerative

Lameness is one of the leading reasons for suffering and ill health in the horse, and it is estimated that approximately 60% of lameness is due to osteoarthritis. Mesenchymal stem cells (MSCs) are a regenerative therapy used to mitigate chronic pain and slow the progression of osteoarthritis. In people, there are FDA non-cross matched allogeneic (non-self) and autologous (self) MSC trials for joint disease and approximately half of all current MSC clinical trials in people are allogeneic.¹ This is because allogeneic MSCs have been long considered non-immunogenic, and are more easily commercialized. This remains true in horses as well, and several groups maintain the feasibility of allogeneic MSC use.^{2,3}

However, as clinical data accumulate, it appears there is greater variability in efficacy of allogeneic therapies as compared to autologous, causing the immune privileged status of allo-MSCs to be questioned.⁴ In horse, published reports document a regenerative effect when autologous MSCs are used to treat joint disease, but anecdotal reports (where allogeneic MSCs are often used) are of minimal treatment effect. The lack of consistent lameness reduction is because allogeneic MSCs are recognized by the immune system, thus precluding the beneficial effects. This is in agreement with our previous work that showed in horse a significant adverse response when allogeneic-MSCs were confined to the intra-articular environment compared to auto-MSCs.⁵

Long considered immune privileged, allogeneic (non-self, from one horse to another) mesenchymal stem cells (MSCs) have enhanced commercialization potential.^{6,7} The immune privileged status of MSCs stems from absent MHCII expression, low MHCI expression, and immunomodulatory action by MSCs. Despite low expression of MHCI, we have recently demonstrated immune recognition of non-self MSCs in horses. This immune recognition may explain the greater variability in efficacy of allogeneic therapies that has been recognized in people and suggested in the horse.^{3,4,8,9} Our recent work, along with data from equine researchers at NCSU demonstrating cytotoxic T cell responses in vitro after allogeneic MSC administration, provides sufficient evidence to question not only the efficacy but also the safety of allogeneic MSCs in the horse.^{10,11}

Despite decades of mesenchymal stromal cell (MSC) therapy, definitive clinical evidence of efficacy does not exist, and it has been postulated that lack of efficacy is due to MSC preparation technique.^{12,13} Supplementation of mesenchymal stromal cells (MSCs) with fetal bovine serum (FBS) has been a standard practice since MSCs were first described in 1970.¹⁴ Since then, numerous veterinary and human medical patients have received MSCs supplemented with FBS for various medical conditions,

including osteoarthritis. Although clinical detection of adverse reactions appears to be rare, xenogen exposure to the MSC recipient because of FBS supplementation can cause local reactions,¹⁵ anaphylaxis,¹⁶ and anamnestic responses,¹⁷ which have been associated with MSC-non response.¹⁸ While immune recognition and resultant adverse reactions are concerning, immune recognition and resultant cytotoxicity against MSCs may be a key factor in the elusive proof of MSC efficacy for most applications including osteoarthritis.^{13,19}

Many blood derivatives including platelet products,²⁰ umbilical cord blood serum²¹ and chemically defined media have been investigated to replace FBS supplementation of MSCs. Based on the ever-decreasing proportion of clinical.trials.gov registered MSC trials, with FBS supplementation of 80% of regulatory submissions in 2014¹ to 40% in 2019, it appears that the human regenerative medicine field agrees that FBS should be replaced. Surprisingly, the veterinary regenerative medicine community has not adopted this stance and FBS supplementation of MSCs remains the industry standard.^{2,3} Similarly, FBS supplementation remains well-accepted in many preclinical MSC investigations in laboratory animals.²² This may be because definitive evidence for immune targeting and cytotoxicity of FBS supplemented MSCs does not exist. Regardless of this disparity in human, veterinary and preclinical fields, it appears that no autologous supplement has replaced FBS with unaltered growth, characterization or clinical efficacy of MSCs.

We have previously shown that 48 hours of FBS depletion is sufficient to mitigate the mild local adverse reaction after repeated intra-articular injection of MSCs supplemented with FBS in horses.⁵ We sought to develop an autologous, xenogen-free method for supplementation of MSCs that would obviate immune targeting of transplanted MSCs by the recipient, by removing FBS for the entire culture period. Our efforts with platelet lysate and platelet releasate, both autologous and pooled, were disappointing as has been previously reported.²³ With platelet products, isolation of MSCs was generally successful, but growth kinetics, cell surface markers, cell morphology and differentiation ability were more variable when compared to the response to FBS supplementation by the same donors.

By developing a replacement to fetal bovine serum supplementation of MSC culture, immune mediated targeting of culture expanded cells will be abolished. By evaluating immune reaction of matched and mismatched recipients we are developing a hierarchy of stem cell immunogenicity, which is a necessary step in pursuit of an acceptable allogeneic donor.

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Stem Cells: what, how, and why?

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Abstract

The use of stem cells clinically has a solid history and also broad undeveloped potential. As we learn more about these cells and their properties, our ability to harness their potential for future benefit will grow. However, there is also significant confusion surrounding discussions of stem cells, due to vague definitions regarding the types of stem cells and controversies that are inherent in the uses of some types of stem cells. This review discusses the types of stem cells, their advantages and disadvantages, and how they can be used clinically and for research. Specifically, the differences between various/common types of pluripotent stem cells is discussed. The utility of pluripotent stem cells compared to lineage restricted stem cells is also considered.

Keywords: Pluripotent stem cells, embryonic stem cells, tissue-specific stem cells, directed differentiation

Introduction

Although there are several kinds of stem cells, in scientific and lay conversations, often the term ‘stem cells’ is used without specificity, which can lead to confusion and misunderstanding. This review focuses on the major types of stem cells, including the types of pluripotent stem cells, as well as lineage-restricted stem cells, their advantages and disadvantages, and their uses in the clinic and in the laboratory.

Types of stem cells

Before discussion of the characteristics of stem cells, their advantages and disadvantages, and their clinical and research uses, it is important to clarify the types of stem cells. In general, there are 3 major types of stem cells: pluripotent stem cells, lineage restricted or somatic stem cells, and totipotent stem cells.

Pluripotent stem cells are the most versatile of stem cells. These cells are named as such because they have the potential to become any cell type that develops from an embryo. Indeed, the classic type of pluripotent stem cell is the embryonic stem cell. Other sources of pluripotent stem cells are somatic cell nuclear transfer stem cells and induced pluripotent stem cells. Beyond the ability to differentiate into any cell type in an embryo, pluripotent stem cell lines are immortal, with unlimited proliferation potential.

Embryonic stem cells

Embryonic stem cells are derived by dissecting and growing the cells that would become the embryo from a blastocyst stage embryo, before any differentiation into germ layers begins.¹⁻³ Human embryonic stem cells drew significant public attention upon publication in 1998,³ because they are generated from human embryos after *in vitro* fertilization. However, embryonic stem cells generated from other animals were in existence for more than a decade before the generation of human embryonic stem cells by the Thomson group. Mouse embryonic stem cells were first published in 1981,¹ and other animal species followed.³⁻⁶

Somatic cell nuclear transfer stem cells

Somatic cell nuclear transfer can also be used to generate embryos for the production of pluripotent stem cells. These cells are generated by removing the haploid nucleus from an oocyte, transferring in the diploid nucleus from a somatic cell, followed by chemical activation of the oocyte.⁷⁻¹¹ Like embryonic stem cells, human somatic cell nuclear transfer cells are highly controversial. First, they are controversial because they can also be used for reproductive cloning, as first publicized by Dolly the sheep.¹² Second, much like human embryonic stem cells, the method involves the production of an early blastocyst from which the pluripotent stem cells are obtained.¹³ Although human reproductive cloning has

not been achieved for ethical reasons, cloned nonhuman primates have been made¹⁴ and thus it is theoretically possible, although inefficient.

Induced pluripotent stem cells

Induced pluripotent stem cells are somatic cells that are induced, through the re-expression of developmental genes, to become pluripotent. As with other types of pluripotent stem cells, this was first achieved in rodent cells,¹⁵ followed by other animals including humans.¹⁵ Because they do not require the use of an embryo or oocyte during their production, their generation and use is less controversial than other types of pluripotent stem cells.

Uses of pluripotent stem cells

Pluripotent stem cells are used for many purposes, some of which were mentioned above. The most well-known uses are the generation of genetically manipulated animals, the production of somatic cells for transplantation, and the production of somatic cells for research.

To generate genetically manipulated animals, pluripotent stem cells are isolated from embryos of the animal of interest, e.g. a mouse. Those cells are genetically manipulated, typically using CRISPR/Cas9 technology. The manipulated cells can then be injected into a blastocyst, which results in the generation of a chimeric animal. If any of the genetically altered cells contribute to the germline, then breeding the animal results in offspring that are genetically altered.¹⁶ This is commonly used to generate research mice to model diseases in the laboratory.¹⁶

One of the most powerful uses of pluripotent stem cells is the ability to direct differentiation into any cell type in the body. In this context, pluripotent stem cells can be expanded into large batches and differentiated into the cell type of interest, which is then used for research purposes. This strategy is particularly important for cells that are postmitotic in adults, such as neurons. Proliferative cells can be obtained from a patient, grown in culture, and investigated; however, if the cell type of interest in the disease is postmitotic, obtaining a sufficient quantity of cells for experimental use is impossible. Examples include neurodegenerative diseases such as Alzheimer's Disease or amyotrophic lateral sclerosis (ALS), where the cell type of interest is the neuron. Live human neurons generally cannot be studied, except through the generation of neurons from pluripotent stem cells. Research in human neuroscience has accelerated exponentially since human pluripotent stem cells were discovered.

The same directed differentiation procedure can be used to generate somatic cells for transplantation, with the caveat that the pluripotent stem cell generation, growth, and subsequent differentiation are done under general manufacturing procedure conditions, which are the practices required to make clinical grade products. However, a significant caveat to the translation of this technology to the clinic is that it is extremely difficult to ensure that differentiation efficiency is 100% before transplantation. Less than perfect efficiency could result in the transfer of undifferentiated cells that result in teratoma formation in the patient.

Pluripotent stem cell characteristics

Pluripotent stem cells have incredible potential, but they are not easy to work with, for several reasons. They require a full knowledge of the starting cell source and ongoing quality control to be sure that they are and remain pluripotent.

Until this point, all the information above has applied to pluripotent stem cells from any source. This implies that embryonic stem cells, somatic cell nuclear transfer cells, and induced pluripotent stem cells are equivalent. However, there are data demonstrating that this is not true. When embryonic stem cells, somatic cell transfer stem cells, and induced pluripotent stem cells are examined side-by-side, there are phenotypic differences among them. Although the exact consequences of these differences are unclear, we cannot assume that all sources of pluripotent cells will perform equivalently in the generation of somatic cells, regardless of whether the final use is research or transplantation.

A key point that needs to be satisfied before using any pluripotent stem cell is to ensure that it is truly pluripotent. There are several levels of stringency that need to be met to prove pluripotency.¹⁸ The

easiest is to look at molecular criteria. For example, cells can be analyzed for the expression of pluripotent genes and the absence of expression of somatic genes.¹⁹ Similar protein profiles can also be examined.¹⁹ However, molecular markers are not perfect and analysis of pluripotent functionality is always preferred. More rigorous is to look at the ability to differentiate into all 3 germ layers using *in vitro* differentiation protocols.¹⁸ The most rigorous method is to truly test pluripotentiality by producing chimeric animals and demonstrating that the pluripotent stem cell contributed to each germ layer plus germ cells.²

Immortality is a key characteristic of pluripotent stem cells; however, this does not imply stability in culture. In fact, karyotypic instability was recognized as a concern early in the use of the cells.²⁰ Improved culture methods have decreased the occurrence of large chromosomal aberrations, but careful analysis demonstrates that with passage, pluripotent stem cell lines change over time, with numerous chromosomal differences, most less than 100 kilobases of DNA in size.²¹ Furthermore, these changes can alter the ability to differentiate into certain somatic cell types.²¹ Thus, careful and constant quality control of pluripotent stem cells is required.

Advantages and disadvantages

In summary, pluripotent stem cells are a powerful tool for medicine and research. Their potency can be harnessed to generate somatic cells for research and for the clinic, as well as to advance our understanding of developmental biology. However, they have some key disadvantages. Most importantly, anything less than completely efficient differentiation could result in development of subsequent cancer in a patient after transplantation.²² Other disadvantages for clinical use include that differentiated pluripotent stem cells for transplantation are not immune matched to recipients and would be rejected without immune system suppression in the transplant recipient.

Lineage restricted stem cells

The other major stem cell type is the lineage restricted stem cell, also referred to as somatic stem cell, tissue stem cell, or multipotent stem cell. These cells are obtained from mature tissues and are the natural source of cell regeneration in tissues during healing or other physiological cell expansions. These cells are typically restricted to differentiation into cells within their own germ layer, or even within their own tissue.

Advantages and disadvantages of lineage restricted stem cells:

The major disadvantage of lineage restricted stem cells is that they are not pluripotent. They can become some cell types in the body, but not all. However, for clinical purposes, they have some significant advantages. First, since they are not pluripotent, they will not form teratomas upon transplantation. Second, they have a long history of clinical use, demonstrating safety and efficacy. Some examples include hematopoietic stem cells, mesenchymal stem cells, and tissue-specific stem cells.

Hematopoietic stem cells have been in use in the clinical setting for decades, particularly in the treatment of cancer. For this purpose, a person's natural hematopoietic stem cells are destroyed and replaced using donated bone marrow, a major source of hematopoietic stem cells.²³ Mesenchymal stem cells are highly versatile, although their differentiation capabilities are controversial. Initial studies demonstrated increased proliferation of mature cell types in many organs, including from other germ layers, after the injection of mesenchymal stem cells, leading to speculation that these cells could be pluripotent.²⁴ Other studies that traced the source of increased cell proliferation after mesenchymal stem cell transplantation demonstrated that the new cells generated do not come from the transplant, but from the tissue, suggesting that mesenchymal stem cells are powerful endocrine modulators that activate endogenous tissue stem cells without directly contributing to the tissue.²⁵

Other tissue specific stem cells are known to exist through their properties: the ability to proliferate to maintain the tissue stem cell reservoir while at the same time providing cells for differentiation into mature cell types. An example is spermatogonial stem cells, which have been

characterized by their properties in the testes, although they are difficult to specifically isolate for study.^{26,27}

Totipotent stem cells

Totipotent stem cells are the least understood and also the least studied, but they are known to exist by their function. These cells distinguish themselves from pluripotent stem cells because they can become any cell type in the body (as pluripotent cells can) as well as the placenta (pluripotent cells cannot).² These cells exist in the very earliest stages of development before the blastocyst forms; however, because they have not been isolated or grown in culture, relatively little is known about their biology.²⁸

Cell fate specification

Given that the uses of stem cells that generate the most interest are for regeneration of somatic cells for clinical and research uses, understanding the tenets of cell fate specification are key to utility of stem cells. Two approaches to cell fate specification are direct reprogramming of somatic cells and use of developmental biology signaling programs for specification.

In direct reprogramming, somatic cells of 1 type are treated with cocktails (genetic, protein, or small molecules) to directly turn 1 cell type into another. This approach skips the intermediate step of creating a stem cell, which makes it relatively fast. The biological methods for this are still being refined, but it has been accomplished for some applications, such as turning fibroblasts into neurons.²⁹ However, the methods are experimental, and it has yet to be done under GMP conditions. Additionally, because it eliminates the intermediate step of creating an immortal stem cell that allows for rapid expansion, it is not ideal when starting material is limited.

The more established approach is to use the known signaling pathways of developmental biology to specify cell fate. This technique can be used starting from pluripotent stem cells, by initiating primitive streak specification, or it can be used on lineage restricted stem cells by initiating maturation of tissue specific progenitors into mature cell types. Pluripotent stem cells can be guided through specification to endoderm, mesoderm, or ectoderm then to mature cells within each germ layer using developmental growth factors or with small molecule analogues that evoke the equivalent cellular signaling response.³⁰ Bioengineered matrices can also contribute to cellular signaling to direct differentiation.

Confirmatory assays

Similar to the confirmation of pluripotency, there are multiple tests of varying rigor for verifying the effectiveness of directed differentiation. The least rigorous is analysis of the gene expression profile, i.e. mRNA from differentiated cells can be analyzed to determine if the expression profile matches that of a somatic cell of the same type that is obtained directly from the tissue of interest. Similarly, but more rigorous is the analysis of the protein profile, demonstrating that the cell not only generated appropriate mRNA, but translated that mRNA to the appropriate protein profile. However, as previously mentioned, functional testing is the gold standard for confirmation of cell specification, demonstrating that the cell functions as and, ultimately, can integrate into the desired tissue.³⁰

Conclusion

Stem cells are fascinating with a solid history of providing clinical benefit and broad applicability for new uses as our understanding of how to harness their potential grows. The applications continue to expand as we learn how to tap into the potential of all that stem cells have to offer.

Conflict of interest

There are no conflicts of interest to declare.

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Follicle growth, oocyte maturation, embryo development, and reproductive biotechnologies in dog and cat

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Abstract

Although the dog and cat both belong to the order Carnivorous, their reproductive physiology is quite different. The dog is a nonseasonal, monoestrus species with spontaneous ovulation only twice a year and with an atypical, postovulatory oocyte maturation. Furthermore, the application of reproductive in vitro biotechnologies such as in vitro maturation (IVM), in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI) or cloning remains quite a challenge in dogs. By contrast, the cat is a polyestrus seasonal species, with ovulation induced several times a year and typical preovulatory oocyte maturation. As a result, manipulation of ovarian physiology is feasible and in vitro reproductive biotechnologies are as efficacious as in cattle. The first part of this review describes the main facts associated with folliculogenesis in the dog and cat (initiation of growth of primordial follicles, appearance of the zona pellucida, formation of antrum, nuclear and cytoplasmic maturation of oocyte, expression of receptors to gonadotrophins, and expression of steroidogenic enzymes). Second part focuses on oocyte maturation, fertilization, and in vivo early embryo development in both species. Last part discusses in vitro reproductive biotechnologies (IVM, IVF, and IVD), embryo transfer, and more recent biotechnologies (cloning, in vitro folliculogenesis, and vitrification of oocytes and follicles). Innovations in the dog are still limited by various characteristics (long ovarian cycle, difficult in vitro oocyte maturation) whereas in the cat, many in vitro techniques are applicable that may also be extended to wild feline species.

Keywords: Folliculogenesis, biotechnologies, embryo transfer, dog, cat

Introduction

Although the dog and cat belong to same order (Carnivorous), their reproductive physiology is quite different. Dog is nonseasonal monoestrus, with spontaneous ovulation only twice a year. Thus, manipulation of ovarian physiology (e.g. to reduce interval between ovulations, to induce ovulation or superovulation, or to produce embryos) is generally difficult. Furthermore, application of reproductive in vitro biotechnologies (in vitro maturation [IVM], in vitro fertilization [IVF], intracytoplasmic sperm injection [ICSI], and cloning) remains quite a challenge in the dog and only a few research teams have mastered them. By contrast, the cat is seasonally polyestrus with ovulation induced several times a year. As a result, manipulation of ovarian physiology (e.g. stimulation or induction of ovulation) is feasible and in vitro reproductive biotechnologies are as efficient as in cattle. Our purpose is to review available information on: 1) main events occurring during in vitro follicle and oocyte growth, in vivo fertilization, and early embryo development; 2) in vitro biotechnologies and embryo transfer; and 3) other biotechnologies.

In vivo follicle and oocyte growth, fertilization, and early embryo development in dog

In vivo follicle and oocyte growth

Contrary to what occurs in many mammals (e.g. mouse, cow, woman, and others), in dog, ovarian follicles appear after birth. Only oogonia are present in the fetal ovary. First primordial follicles (oocyte surrounded by granulosa cells) appear ~ 1 month after birth.¹ These follicles make up the definitive stock of oocytes. Some of them will start growing only years later. When growth starts, oocyte diameter

increases and granulosa cells appear modified (rounded/cuboidal). As they increase in number, first follicles with an antral cavity appear at 4 months of age. As an antrum is formed, granulosa cells differentiate, distinguishing external cells, close to basal membrane from cells in the cumulus, close to the oocyte. As in other mammalian species, a number of changes take place as the oocyte and follicle increase in size. Changes (Figure 1) include formation of zona pellucida around oocyte, multiplication of granulosa cells (200 - 400 cells when antrum appears),⁸ and first cells in theca, expression of FSH and LH receptors and ability to produce steroids (steroidogenic enzymes).

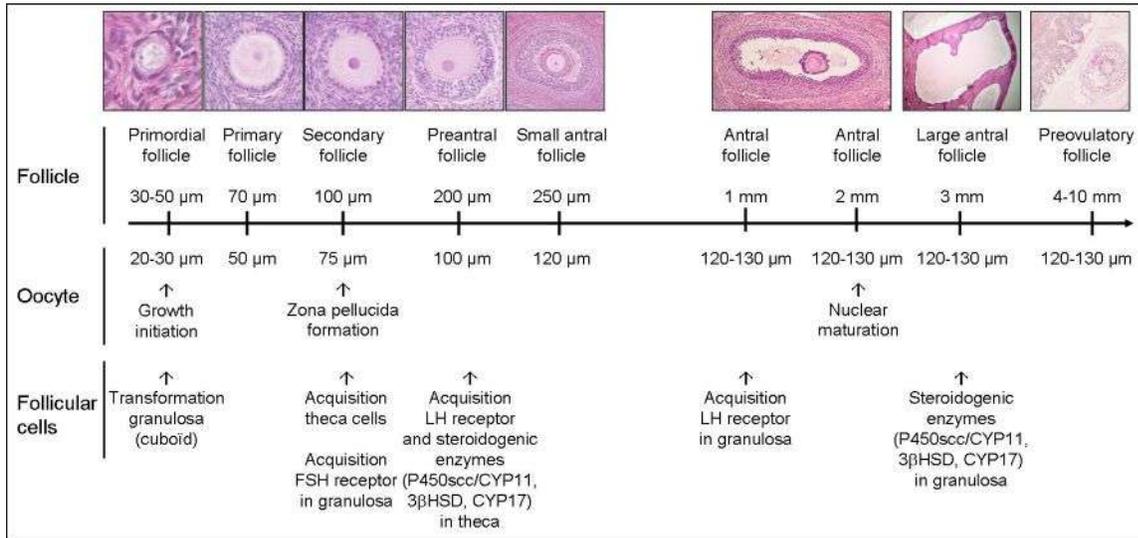


Figure 1. Main events in dog folliculogenesis.¹⁻⁷

During anestrus, some follicles reach 1 mm in diameter.¹ Oocyte is surrounded by granulosa cells of cumulus, with many contacts between them and oocyte, leading to continuous transfer of many compounds (pyruvate, growth factors, RNAs, and organelles) from somatic cells to oocytes (Figure 2).

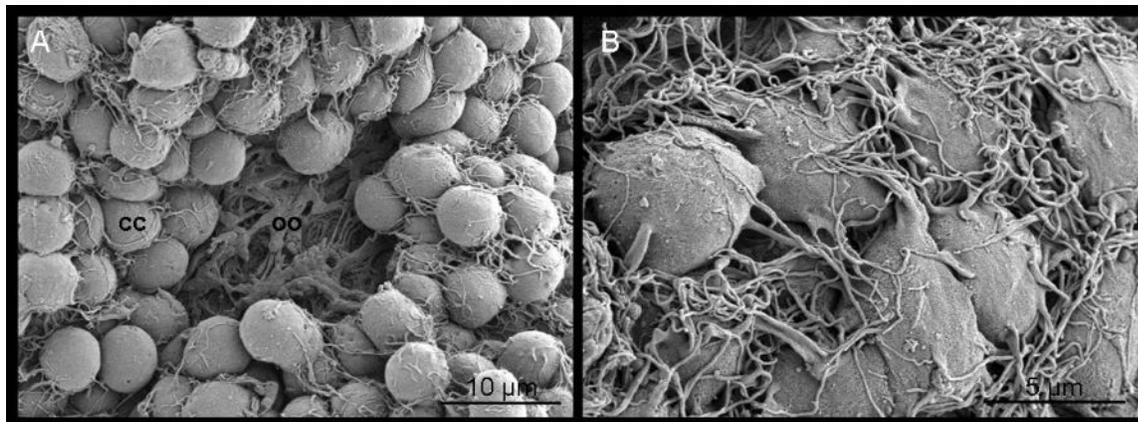


Figure 2. Scanning electronic microscopy image of a dog cumulus-oocyte complex showing numerous "pseudopod-like" connections between the cumulus cells (cc) and the oocyte (oo) (A) and between the cumulus cells (B).

Inside a follicle, the oocyte gradually acquires its ability to resume meiosis (nuclear maturation) and later acquires ability for fertilization and early embryo development (cytoplasmic maturation). A unique feature is the number of follicles with multiple oocytes (polyoocytic follicles) in the ovaries of dogs and

cats.^{1,9-11} In dog, as many as 14% of follicles have several oocytes (with a maximum of 17 oocytes in 1 follicle) while it represents < 1 - 2% in other mammals.¹² They may reach ovulation but represent only 4 - 7% of preovulatory oocytes. Among them, a single oocyte in the follicle appears to be of good quality.¹³ After puberty, at each cycle, larger follicles with antrum are being recruited to initiate terminal growth. Growth of this follicle may be visible by ultrasonography towards the middle and end of anestrus.¹⁴ Once estrus begins, follicle growth accelerates and under stimulation by LH at the preovulatory stage, follicle wall becomes thicker. Immediately prior to ovulation, a protuberance may be visible at the very site where the oocyte is released (Figure 3). Right after ovulation, follicles are transformed into corpora lutea.

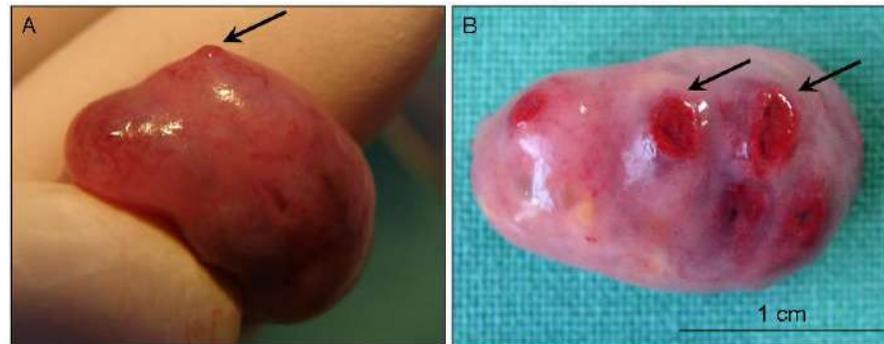


Figure 3. Dog ovaries during periovulatory stage, site (arrow) of future oocyte expulsion is visible immediately before ovulation (A), whereas small haemorrhagic corpora lutea (arrows) are visible right after ovulation.

Monitoring ovulation in dogs is possible by various means¹⁵ such as LH or endocrine assays, possibly coupled with ultrasonography, depending on the veterinary clinic. Indeed, as a result of preovulatory luteinization of ovarian follicles, blood progesterone concentrations gradually rise from basal concentrations to 2 ng/ml at LH peak and 3 - 10 ng/ml at ovulation.¹⁶⁻¹⁷ Thus, it is possible to predict ovulation and have the followup in real time by ultrasonography.^{18,19} With 2 ultrasonographies a day, disappearance of follicles can be confirmed. However, not all follicles will transform into corpora lutea; some become filled with blood right after ovulation and may thus appear anechogenic. Follicle size at ovulation is related to dog breed and may vary from 4.5 mm in small breeds to 8 - 10 mm in larger breeds.^{20,21}

At ovulation, oocytes are surrounded by mucified granulosa cells and are drawn into the infundibulum to enter the oviduct, but are still unfertilizable. Then, the oocyte loses the external layers of cumulus and migrates rapidly to middle section of oviduct (total length, 5- 10 cm). Several important events take place in oviduct: resumption of oocyte meiosis, selection of sperm,^{22,23} and initiation of embryo development.²⁴ Resumption of meiosis leads to first polar body expulsion (2 - 3 days after ovulation) from oocyte leading to metaphase II stage that is ready for fertilization.^{25,26} At that stage, it becomes possible to collect in vivo-matured oocytes. Two research teams have been able to routinely collect oocytes in vivo and have achieved in vitro fertilization and generated genetically modified animals or clones.²⁷⁻³⁰ Ability to reach the fertilizable metaphase II stage after extraction from the follicular environment increases from 17 to 80% for oocytes from small follicles (< 500 μ m) versus those from larger follicles (~ 2 mm).⁷ Maturation of oocyte cytoplasm is more difficult to investigate, as it would require examination of oocytes from various follicle sizes (e.g. 2- 4, 4 - 6, and 6 - 8 mm). After fertilization, these oocytes should be examined through fertilization and embryo development until blastocyst stage. Several papers reported results obtained with oocytes from large follicles.^{27,31} Unfortunately, in vitro culture conditions are still suboptimal, so that the exact time of cytoplasmic maturation acquisition remains uncertain.

As mentioned earlier, blood concentrations in progesterone in dog at ovulation and shortly thereafter are extremely high (> 10 times those in cattle or women). Presumably, these high concentrations of progesterone have significant biological effects. Thus, progesterone receptors (e.g.

aglepristone) treatment several days before ovulation induced a delay in resumption of meiosis and increased oocyte survival. It also inhibited the upward movement of sperm in oviduct.³² Since oocytes are not readily fertilizable at ovulation, artificial insemination (AI) of thawed sperm, in order to obtain embryos or a pregnancy, should be done up to 2 days after ovulation. By contrast, in natural breeding, sperm survive much longer (up to 11 days),^{25,33} making much earlier breeding before ovulation possible. Following fertilization, embryo development not only relies on oocyte reserves, but also requires exchanges with the environment in oviduct to obtain metabolites needed for further development. Hormone assays, coupled with ultrasonography, made it possible for us to describe precise timing of embryo development after AI in the dog^{26,34} (Figure 4 and Table). Following morula stage, 8.5 - 9 days after ovulation, embryos cross utero-oviductal junction and start uterine entry.³⁵ Prior to implantation, embryos migrate frequently to uterine horn on opposite side³⁶. Implantation takes place ~ 17 days after ovulation.³⁷

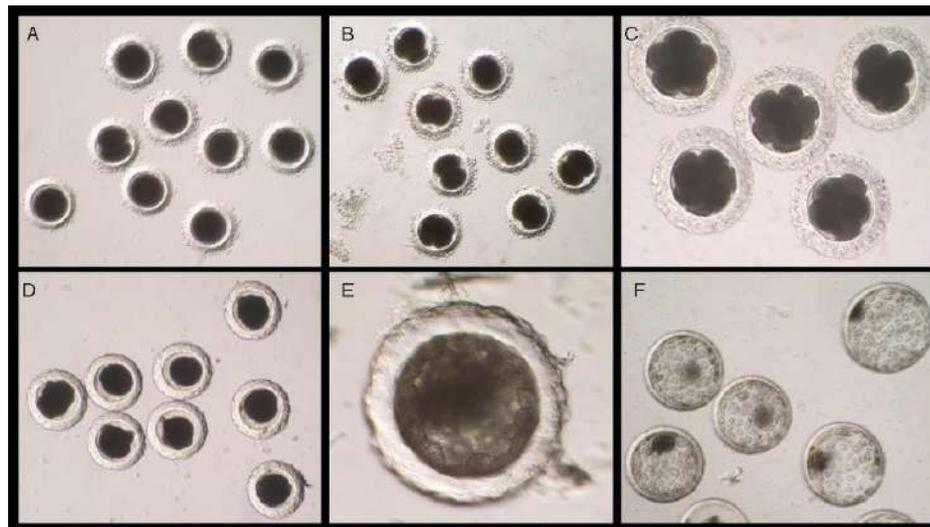


Figure 4. Dog embryos collected *in vivo* at 2 pronuclei (A), 2 or 4 cell (B), 8 cell (C), morula (D), early blastocyst (E), and expanded blastocysts (F) stages.

		Stage of embryonic development					
		2-PN	2-cell	4-cell	8-cell	Morula	Blastocyst
	n embryos (n bitches)	37 (7)	33 (8)	12 (5)	34 (7)	22 (4)	22 (5)
Time after ovulation	hours	102- 132	120- 161	133- 154	153- 225	230- 266	230-274
	days	4.5-5.5	5-7	5.5-6.5	6.5-9.5	9.5-11	9.5-11.5

Table. Timing of embryo development *in vivo* in the dog (note: fertilization takes place 2-3 days after ovulation)

Challenges to *in vitro* maturation, fertilization, and embryo development

In vitro oocyte maturation and fertilization in dog have been investigated for several decades³⁸ and many obstacles were encountered. However, major progress has been achieved in the last 20 years. Reproductive *in vitro* biotechnologies represent an important research tool in that they may elucidate the biological mechanisms of oocyte maturation, fertilization, and embryo development, in particular the respective role of environmental factors (e. g. endocrine control, metabolism, growth factors, proteins, and intercellular communications). These biotechnologies also make it possible to preserve genetic resources (e.g. freezing of oocytes, sperm, embryos, ovarian cortex, and testicular pulp) for valuable dog

models in biomedical area (refer Online Mendelian Inheritance in Animals website [OMIA.org] that lists animal models for human diseases, 450 canine models) or to preserve threatened wild canid species.

In vitro maturation of dog oocytes

Purpose of in vitro oocyte maturation (IVM) is to obtain a large number of fertilizable oocytes. After ovariectomy and careful dissection of ovary, oocytes are selected and cultured in vitro in a maturation medium, using bovine models. In other mammals, when oocytes are collected from ovaries/follicles, there is an immediate resumption of meiosis in all competent oocytes (oocytes that reached a sufficient degree of nuclear maturation). For example, in cattle and mice, > 80% of oocytes have resumed their meiosis and become fertilizable after 24 hours. In dog, by contrast, oocyte reaches metaphase II stage and thus becomes fertilizable after 3 - 4 days in culture and with < 20% yield.³⁹ As mentioned above, folliculogenesis and thus oocyte maturation within the follicle is a process lasting for months. Final folliculogenesis, at ovulation, makes it possible to collect good quality oocytes but takes place only twice a year in dog. Collecting oocytes from an ovary during anestrus results in oocytes that are still quite immature. Furthermore, ovaries collected by ovariectomy may still be at prepubertal stage, that is 2 - 3 months only after appearance of first follicles with antrum. These oocytes are even more immature than anestrus oocytes, with poor synthesis of proteins and limited connections with granulosa cells.⁴⁰

Many research teams have attempted to produce in vitro-matured fertilizable canine oocytes and to identify oocytes that should be used (according to age, breed, stage of cycle), to determine which media, oxygen supply, growth factors, proteins, hormones should be introduced and which cells to use in co-culture (reviewed^{39,41,42}). Overall, the results have been disappointing. Major problem appears to be oocyte size compared to follicle size.⁴³ Thus, using oocytes from large follicles makes it possible to increase the proportion of fertilizable oocytes after in vitro maturation.⁴⁴ Yet, rates of success remain low, which may be related to cytoplasm quality (e.g. number and organization of organelles such as mitochondria and cortical granule) in those in vitro-matured oocytes reaching the metaphase II stage, which remain largely below those obtained with oocytes ovulated in vivo.⁴⁵

Current investigations attempt to mimic in vivo intraovarian environment with maturation in pure follicular fluid⁴⁶ or in diluted follicular fluid,⁴⁷ or within an oviduct. Research on oviductal environment, which started 20 years ago,⁴⁸ is now designed to determine the composition of oviductal fluid and physiology of cells in oviductal epithelium^{31,49,50} since exposure of oocytes to oviductal environment is essential for normal fertilization.⁵¹ Variety of culture conditions have been tested such as synthetic oviductal fluid medium⁵², use of microvesicles produced by oviductal cells,^{50,53} cell cultures on plastic dishes or porous inserts,⁴⁹ oviductal explants⁵⁴ (fragments of oviductal epithelium), or ligated oviduct.⁵⁵ However, maturation rates hardly reach 20% and canine oocytes collected during anestrus, even transferred in vivo into an oviduct of dogs in estrus, are not able to adapt, despite having a potentially optimal environment.³⁴

Some investigations were conducted in cattle, to increase the rate of metaphase II oocytes and primarily the rate of fertilization and embryos at the blastocyst stage (currently reaching 30 - 60%). These investigations were designed to temporarily block meiosis resumption, right after rupture within the follicle, in order to give the oocyte a chance to complete its cytoplasmic maturation. Several compounds such as roscovitine and dbcAMP were tested in anestrus canine oocytes without significant success.^{57,57} Thus, the problem of immaturity of anestrus oocytes is far from being solved. As a result, up to this day, a single early pregnancy was reported after IMV-IVF-IVD⁵⁸ but no puppy was born. Birth of pups following embryo transfer (from oocytes, matured in vivo, and fertilized in vitro) was a major progress.²⁷

In vitro fertilization and embryo development

In vitro fertilization is now routinely carried out in a number of species. Progress in reproductive biotechnologies in dog is in part from applying results obtained in wild canids (e.g. silver fox). Large numbers of ovaries collected on farms rearing foxes for fur production made it possible to describe maturation of oocytes (ovulated at an immature stage like in dog), in vitro fertilization and transfer.⁵⁹⁻⁶²

Altogether, rates of *in vitro* maturation and fertilization in the silver fox are close to those obtained in dog. Surgical embryo transfer has led to the birth of progeny.⁶³ However, *in vitro* fertilization and embryo development remain problematic in the dog, due to a number of causes. Cytoplasm quality of canine oocytes is generally poor, zona pellucida undergoes changes during culture and sperm capacitation has not been perfected.⁶⁴⁻⁶⁵ Microinjection of sperm into oocyte (or ICSI), allowing a normal monospermic fertilization, has been reported in dog, however, is not routinely used in laboratories.^{66,67}

Several teams have described *in vitro* fertilization and occasionally obtained embryos and even puppies.²⁷ However, bringing together dog sperm and oocytes *in vitro* does not routinely lead to successful fertilization, because sperm can enter immature oocytes,^{64,68} typically ending in a high rate of polyspermia^{44,69} and sperm pronucleus abnormalities.^{37,65} However, oocytes collected at follicular phase stage have been used successfully to obtain embryos with as many as 30% undergoing cleavage after fertilization using oocytes from follicles > 2 mm.⁷⁰ Following fertilization, embryo development is initiated; however, it is blocked at the 8 cell stage when the embryo becomes autonomous and develops its own gene expression (stage of maternal to zygotic transition).⁷¹ Blocking at 8 cell stage suggests that conditions of embryo culture are still not optimal. However, it appears that co-culture with embryonic fibroblasts may improve development of canine embryos to 16 cell/morula stages⁷² and that some recently described culture media might enable development of embryos up to the blastocyst stage.^{73,74}

In vivo collection, freezing, and transfer of canine embryos

As stated above, *in vitro* production of canine embryos is quite problematic. Another possibility is to produce *in vivo* embryos after ovulation, followed by natural breeding or artificial insemination (reviewed^{69,75}). Collecting embryos *in vivo* may be achieved by flushing the oviduct to collect embryos at an early stage (2 cell - morula) or rinsing uterus to obtain embryos at morula and blastocyst stages. A major difficulty remains in getting access to oviduct without ovariectomy, since oviduct is hidden in a lipid-rich ovarian bursa and infundibulum is difficult to handle. By contrast, collecting embryos in uterus is simple and requires only a laparotomy, followed by uterine rinsing.^{76,77} It may also be achieved by a nonsurgical endoscopic technique, with the introduction of a suitable catheter into cervix, which may allow to keep the female donor as a reproducer.^{78,79} Embryos obtained this way may then be transferred to a recipient female or can be frozen. Canine embryos from 1 donor female are difficult to obtain in large numbers, as well *in vitro* (as described above) as *in vivo* because female dogs do not respond to superovulation treatments and because the number of embryos obtained is limited by ovulation rate.

Pioneering research in canine embryo transfer was started in the '80s.^{25,76,80,81} Transfer of fresh embryos required optimal synchronization between donor and recipient cycles. At that time, method to synchronize cycles was not available, hence many experimental kennels were needed to routinely obtain females in estrus that could be used as embryo recipients. Ten years ago, only 45 puppies were born in the world after transfer to 57 recipient females (reviewed⁶⁹). Since then, a number of puppies were born after oocyte micromanipulation (cloning), and after IVF and transfer.^{51,82} Currently, estrus control, suppression or stimulation, can be obtained using GnRH agonists. These treatments are efficient and free from negative impacts on subsequent fertility.⁸³⁻⁸⁵ Thus it becomes possible, towards end of anestrus period, to induce estrus in a dog within a few days.⁸⁶ Generally, embryo is transferred into uterus because oviductal access is difficult. Even at oviductal stages (2 - 16 cells), embryos can be successfully implanted in uterus.

In the absence of efficient synchronization treatment, cryopreservation of embryos may allow postponement of embryo transfer until a recipient female is available. Conditions for optimal cryoconservation of dog embryos remain an area of investigation, including: duration of exposure to cryoprotectants and concentration of cryoprotecting agents, evaluation of cryotolerance beyond morphological evaluation and conditions of embryo transfer after freezing and thawing to obtain pregnancies remain to be well defined.

Two freezing processes are used, namely, slow freezing and vitrification. Regarding slow freezing, glycerol and ethylene glycol were evaluated and gave contradictory results. In a study involving 20 embryos, it appeared that glycerol alone was able to preserve zona pellucida structure.⁸⁷ However,

similar post-thaw viability was established in glycerol and ethylene glycol when 50 blastocysts were used.⁸⁸

In vitrification, high concentrations of cryoprotectant agents are being used, embryos are placed in a 1 - 5 µl microdrop and lowering of temperature is very rapid, with direct immersion in liquid nitrogen. This ultrarapid freezing is designed to avoid crystal development within cells and appeared proper for dog embryos. Thus, after vitrification in 1 µl microdrops in a Cryotop system, early canine embryos (zygote to 16 cells) survived freezing better (90 - 100% of survival after thawing) than embryos at morula (50%) or blastocyst (40%) stages.⁷⁸ These investigators also transferred 77 embryos to 9 recipient females and obtained 5 pregnancies, 4 to term, and 7 puppies were born. A lag time of 1 or 2 days between donor and recipient cycle stage of the donor was not a deterrent to pregnancy.⁷⁸ Furthermore, a recent study⁸⁹ compared slow freezing and Cryotop method vitrification in 89 *in vivo* collected embryos at various stages (8 cell to blastocysts). After cryopreservation (30 embryos by slow freezing and 35 by vitrification) and surgical transfer in recipient females (1 - 6 embryos per recipient), they succeeded in obtaining 2 pregnancies (1 puppy per recipient), but only with vitrified embryos.

Reproductive biotechnologies under development for preservation of dog genetics

Somatic cloning

Somatic cloning, also termed somatic cell nuclear transfer, which consists of injecting somatic cell into a mature oocyte, was first described in the dog in 2005.²⁸ Indeed, the first dog obtained by cloning, Snuppy, a male Afghan hound, was re-cloned recently.⁹⁰ Since then, the Korean research team has specialized in the collection of mature oocytes followed by micromanipulation. Right after transfer of nucleus, potentially cloned embryos are transferred into an oviduct using an appropriate catheter (Tom cat 3.5Fr) in order to avoid blocking of canine embryos in culture at 8 - 16 cell stage.⁹¹ The technique has been considerably improved and its yield in number of puppies born related to the number of injected oocytes has made progress. South Korean investigators have applied cloning to a number of situations: pet dogs, working dogs (e.g. scent detection dogs), preservation of breeds (e.g. Sapsaree and the Gyeongju Donggyeong dogs), preservation of wild species (e.g. grey wolf and coyote), not to mention the creation of transgenic and canine biomedical models of human disease (reviewed⁸²). Currently, it is possible to obtain cloning of a dog or a cat in South Korea, in China or in US for approximately 30,000€ per cat and 45,000 - 50,000€ per dog. Thus, the Sinogene company in Beijing reported that it had cloned as many as 40 dogs and cloned its first cat in 2019. Similarly, the South Korean Sooam company reported it had cloned 800 pet animals. In parallel, the US company Viagen Pets also offers cloning.

Cryopreservation of ovarian cortex/oocytes and *in vitro* folliculogenesis

Some other reproductive biotechnologies could be used for purposes of fundamental research or for preservation of biodiversity. *In vitro* folliculogenesis (dissection of ovarian follicles followed by culture for several days or weeks) may answer a number of questions regarding the growth of follicles and oocytes.⁹²⁻⁹⁵ For instance, in mice, one may, over an interval of 3 weeks, culture primary follicles until the preovulatory stage and obtain newborns.⁹⁶ However, in dog, as in large mammals, folliculogenesis is a very lengthy process and culturing follicles for months remains quite a challenge and no progeny has been obtained so far anywhere in the world. However, in women, cryoconservation of the whole ovary or of fragments of ovarian cortex followed by autografting has been successfully used to preserve fertility prior to some gonadotoxic anticancer therapy. Following the termination of cancer therapy, the ovary or cortex fragments can be reimplanted and can restore ovarian function, leading to growth of the uterus and eventually pregnancy and the birth of a baby. This procedure for fertility preservation (freezing + grafting) has successfully generated dozens of babies.⁹⁷ In dogs, these techniques are still in an experimental stage. However, canine follicles survived in frozen ovaries and autografting was possible with resumption of follicle growth.⁹⁹ Another way to preserve female genetic material is cryoconservation of oocytes. In humans, this technique is widely used since the development of

vitrification. Two attempts of vitrification have been reported in dogs. Freezing immature oocytes has already been tested and 65% of the cumulus-oocyte-complexes were reported to have adequate morphology after vitrification by the Cryotop method.¹⁰⁰ Similarly, wolf oocytes survived vitrification.¹⁰¹

Further studies are needed to examine oocyte survival and developmental potential after vitrification, according to the presence of cumulus cells and maturation stage at cryopreservation. Research in this area may benefit from the results of vitrification in pigs,¹⁰² whose oocyte is also rich in lipids, like that in the dog. Another technique commonly used in cattle, *in vivo* follicle puncture, also called Ovum Pick Up (OPU), may be quite useful in dogs. It is feasible in dog; however, it requires having a dog at similar preovulatory stage, good ultrasound equipment to clearly visualize follicles, and a laboratory close by to manipulate the oocytes (observation, culture or micromanipulation). Following OPU, oocytes or cumulus-oocyte-complexes might be cryopreserved or kept for maturation.

In vivo follicle and oocyte growth, fertilization, and embryo development in cat

Similar to dogs, a cat ovary starts developing during fetal life (oogonia migration/oogenesis); however, first primordial follicles appear approximately 1 month after birth.¹⁰³ Histology of follicle and oocyte growth have been reported.¹⁰³⁻¹⁰⁶ Primordial follicles, primary, secondary, and preantral follicles measure 50, 80, 130, and 150 μm respectively and contain an oocyte whose size is 40, 60, 90, and 100 μm respectively.¹⁰⁶ Zona pellucida appears in secondary follicle and antral cavity appears when follicle reaches 220 μm .¹⁰⁶⁻¹⁰⁷ At that stage, oocyte reaches 110 μm (together with zona pellucida) and nuclear maturation is completed. At the time of ovulation, oocyte size is \sim 125 - 130 μm .¹⁰⁸ Receptors for FSH in granulosa cells and for LH in theca cells can be detected in 200- μm small antral follicles.¹⁰⁹ In granulosa cells, LH receptors appear when antral follicles reach at least 800 μm . Similar to dog, cat ovary contains a large number of polyoocytic follicles, \sim 4%, with a recorded maximum of 10 oocytes in a single follicle.^{9,103,110} Preovulatory diameter is 3 - 4 mm and may be measured via laparotomy or by laparoscopy.^{111,112} Ultrasonography may be used^{113,114} without anaesthesia, particularly, in docile animals. Regular assessment of behavior, vaginal smears and/or ultrasonography is needed, however, as these parameters are poorly correlated.¹¹⁴ Ovulation can be induced by vaginal stimulation, inducing a peak in LH concentration, or may be obtained by hCG injection. Ovulation may also be spontaneous, without coitus.¹¹⁵ Meiosis resumption resumes within ovary and fertilization may take place as soon as oocyte reaches oviduct. After *in vivo* fertilization, embryos at 1 - 4, 5 - 8, 9 - 16 cell, and morula stages were present in oviduct \sim 28 - 34, 40- 46, 64 -70, and 88 - 94 hours respectively after ovulation.¹¹⁶ Beyond 112 - 118 hours postovulation, compact morulas and blastocysts were present in uterus.¹¹⁶ Prior to implantation, embryos migrate frequently to uterine horn on the opposite side.¹¹⁶

In vitro oocyte maturation, fertilization, and embryo development in cat

Contrary to dog, cat is a species in which oocyte maturation, fertilization and *in vitro* embryo development can be achieved with a rate of success similar to those obtained in cattle. Thus, already more than 30 years ago, the first cat embryos were obtained after *in vitro* fertilization (but with *in vivo* matured oocytes).^{108,117} Using oocytes collected from 55 female cats after ovarian stimulation (PMSG + hCG) made it possible to obtain as much as 80% of matured oocytes and 35 - 45% fertilization rate, with $<$ 5% polyspermia.¹⁰⁸ After transfer of embryos into oviduct following laparotomy (54 embryos in 5 recipient cats), 4 litters and a total of 10 kittens were born. Pregnancy rates were satisfactory after transfer of embryos into uterus. However, embryo survival and thus the number of kittens obtained were higher after transfer into an oviduct.¹¹⁸ Furthermore, using *in vivo* matured oocytes, the rate of embryo development was high: out of 100 oocytes, 75% divided after *in vitro* fertilization, 66% developed to the morula stage, and 18% to blastocyst stage.¹¹⁹

Even with oocytes collected with no hormonal stimulation after ovariectomy, results can be favourable. Thus, $>$ 45% of oocytes reach metaphase II stage after 32 - 38 hours of maturation,¹²⁰ and after *in vitro* fertilization (46 - 56% of fertilization rate), 30 - 40% of embryos develop.¹²¹ Furthermore, *in vitro* development is similar to *in vivo*.¹²² However, since cat is a seasonal species, further testing was carried out to evaluate impact of period of the year on developmental competence of oocytes used for *in*

vitro fertilization. In a study¹²³ on nearly 7000 cumulus cell oocyte complexes, a seasonal effect was observed, with 45 - 55% embryos cleaved after fertilization and 50 - 70% of these embryos developing up to the blastocyst stage, but with lower rates of success in fall and winter seasons (October - March). In ICSI, even with epididymal or testicular sperm, 37% of embryo developed to morula stage and some births of kittens were reported.¹²⁵ After cryoconservation of testicular tissue followed by microinjection of sperm into oocytes matured in vitro, embryos could be obtained, which were then frozen and transferred to several females, leading to birth of several kittens.¹²⁶ Ease of assisted reproductive technologies achievement in cat has led to research on wild felids (reviewed¹²⁷⁻¹³⁰). For example, 30 years ago, embryos of a leopard cat (*Felis bengalensis*) were obtained after ovarian stimulation by PMSG + hCG treatment followed by follicle puncture via laparoscopy, in vivo fertilization and early embryo development.¹³¹ Very recently, the first Cheetah pups were born as the result of in vitro fertilization and embryo transfer achieved by scientists of the Smithsonian Conservation Biology Institute (<https://www.si.edu>). Some other teams, such as that of Bill Swanson (Cincinnati Zoo and Botanical Garden), have become real experts in followup/induction of ovulation in domestic cats and also wild felids. This research was applied to the production of animals in zoos and in the wild, to building genetic stocks of sperm and embryos and to maintenance of animals of biomedical interest. Indeed, as in dog, a number of feline models can be used in biomedical research (OMIA.org, 218 feline models of human disease), and the international conference on canine and feline genetics and genomics is devoted to these specific models.

Other reproductive biotechnologies for the preservation of cat genetics

Somatic cloning

The first cat obtained by somatic cloning, "CopyCat", was born in 2001.¹³² Genetics Saving and Clone company started its business and produced a first cat, "Little Nicky" sold for \$50,000 in 2004. This company kept producing cloned cats until 2006. In the world, cat cloning can be done in South Korea, in China or in US (refer above, section "dog cloning"). A cloned cat can, as the dog, be re-cloned.¹³³ Furthermore, numerous attempts at interspecific cloning were carried out. Somatic cells of wild felids were injected into oocytes of domestic cats and some small wild felids (e.g. African wild cat¹³⁴) were obtained this way (reviewed^{135,136}).

Collection and preservation of the female genetic potential

In vitro folliculogenesis in the cat was also explored. It is a powerful tool to study biological mechanisms and one way to stock genetic potential in a biobank.^{137,138} Indeed, preantral follicles are quite numerous in ovaries and can survive cryopreservation.¹³⁹ Cryoconservation of immature cat oocytes by vitrification with the Cryotop system has been described.¹⁴⁰ High rates of oocyte survival were reported with resumption of maturation after thawing in ~ 25 - 40% of oocytes. After ICSI (freezing may alter zona pellucida, and thus prevent fertilization), as many as 20% of embryos may reach the morula stage. Some kittens could be obtained using vitrification technique.¹⁴¹

Conclusion

Altogether, despite the complexity of canine model (2 ovulations per year, limited number of mature oocytes available and requirement of an experimental kennel), research efforts in reproductive biotechnologies are in full development, with the efforts of a few research teams in the world. On the contrary, in cat, numerous in vitro techniques are applicable that may also be extended to wild feline species. Development of worldwide communications gave the scientific community a chance to collaborate. Thus international embryo technology society created a committee on companion animals, nondomestic, and endangered species with the objective of sharing research progress in this area (https://www.iets.org/comm_candes.asp).

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“This stud’s a dud!”- Canine semen evaluation protocols and pitfalls

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Abstract

Properly performed canine semen evaluation is a major component of breeding soundness examination. Various types of equipment and protocols are available to practitioners and laboratory technicians to accurately assess sample concentration, motility and morphology. However, it is vital that precise laboratory standardization and quality control procedures be followed to optimize accuracy and usefulness of a semen evaluation.

Keywords: Semen evaluation, canine, dog, breeding soundness examination

Applications of a semen evaluation

Importance of an accurately performed semen evaluation as part of a breeding soundness examination cannot be over emphasized. Results are used to monitor dogs in a breeding program and those undergoing therapies for subfertility or infertility. Complete semen evaluation should always be done as part of pre-purchase examination or evaluation of chilled or frozen semen for artificial insemination.

No single parameter from semen evaluation that practitioner can use to accurately assess fertilizing capability of a dog or predict outcome of a successful litter.^{1,2} However, breeding recommendations to maximize likelihood for successful pregnancies are possible using dependable and precise semen evaluation results.

Breeding soundness examination and paperwork

Obtaining a detailed history and performing a thorough physical examination prior to collecting a dog is vital, as there are many outside factors that may affect quality and accuracy of semen that might otherwise be overlooked.³ Society for Theriogenology (SFT; www.therio.org) has a comprehensive breeding soundness examination form (refer next page) to record reproductive history, pedigree, physical examination and semen evaluation, to promote consistency and accuracy.

Laboratory setting

Prior to collecting dog, microscope stage, slides and coverslips should all be pre-warmed to 37 C° to avoid any temperature shock when transferring samples for evaluation.⁴ Correct labeling of all slides, test tubes and containers used in evaluation with a permanent marker is essential for record accuracy.

Semen analysis reliability

World Health Organization laboratory manual recommends standardized, evidence-based procedures for human semen analysis to improve reliability, accuracy and comparability of results from laboratories.⁵ Unfortunately, these protocols are deficient in most canine commercial laboratories and private practices. This is a dilemma not only for researchers trying to analyze data, but also for practitioners depending on semen analysis accuracy for breeding recommendations or to monitor success of canine reproductive therapies.

Semen volume

Volume of ejaculate collected is not correlated to semen quality, but is required to calculate semen concentration,² so must be accurately recorded prior to starting any laboratory testing. Volume of first fraction varies from 1 - 5 ml and second fraction from 1 - 3 ml.



CANINE BREEDING SOUNDNESS EVALUATION

Guidelines Established by the Society for Theriogenology
761 Tiger Oak Drive, Pike Road, AL 36064-3063 • (334) 395-4666

AKC Reg #/Other ID # _____ Exam Date: _____
Call Name: _____ Registered Name: _____
Client Name: _____ Address: _____
Breed: _____ Color: _____ Date of Birth: _____

HISTORY

Reason for evaluation: _____
Date of last litter: _____ Brucellosis test: positive/negative Date of test: _____ Test used _____
Infertile relatives: _____

PHYSICAL EXAMINATION

Body condition: _____ Weight: _____ pounds/kilograms
Pertinent health problems: _____
Penis/Prepuce: _____ Scrotum: _____
Prostate: _____
Epididymides: (R) _____ (L) _____
Testes Width: (R) _____ (L) _____ Spermatic cords: _____
Testes Consistency: (R) _____ (L) _____ Masses/fluid/pain/other: _____

SEMEN COLLECTION & EVALUATION

Libido/Ease of collection: Poor / Fair / Good / Excellent Teaser bitch present: Yes/No Stage of cycle: _____

	Color	Volume (ml)	Concentration (sperm/ml)	Total Sperm/Ejaculate
Fraction 1	_____	_____	_____	_____
Fraction 2	_____	_____	_____	_____
Fraction 3	_____	_____	_____	_____

Total Motile Spermatozoa: _____% Progressively Motile Spermatozoa: _____% Speed/velocity of motility: 0-5 _____

Morphology: Stain(s) utilized: _____
% Normal: _____ Number Cells Counted: 100/200
Head defects (%): _____
Midpiece defects (%): _____
Tail defects (%): _____
Other defects (%): _____

Longevity: Extender(s) used: _____ Diluent rate: _____
Motility: 24 hours _____ 48 hours _____
Cytology: Fraction(s) evaluated: _____ Stain(s) utilized: _____
Presence of RBC, WBC, Epithelial cells, Bacteria, Germ cells (0 – 4+): _____

CONCLUSIONS / COMMENTS / INTERPRETATION

Signed: _____ Clinic Name: _____
(Member – Society for Theriogenology) Address: _____

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Semen color

Subjectively, second fraction is normally opalescent. Blood causes a pink or red discoloration possibly from either prostatic disease or penile trauma. A yellow color could indicate urine contamination or riboflavin (often present from dogs on raw diets) whereas no color often is an indicator of few to no sperm in the sample. A green hue could indicate an inflammatory exudate or excess smegma.⁶

Semen pH

Validity of this parameter is controversial and it is not on the SFT breeding soundness evaluation form. Unfractionated canine semen pH ranges from 6.4 - 6.8. Inaccuracy of pH paper strips along with newer antibiotics that ionize at multiple pH values decreases pH testing validity in dogs.⁷

Semen motility

Evaluation of sperm motion cannot be used to accurately predict fertilizing potential of sperm in a given sample when used for artificial insemination.⁸ Motility evaluation should be done as soon as possible after semen collection.⁹ There are 2 main methods for evaluating progressive motility, which normally should be $\geq 70\%$ for dog sperm.

First method is a subjective evaluation of semen motility and involves assessing a drop of semen flattened between a microscope slide and a coverslip. Normal sperm should traverse microscopic field in $\sim 2 - 3$ seconds in a forward progressive movement.² Motility is best assessed using a properly aligned phase contrast microscope at 250X magnification.¹⁰

Layer of sperm should be only 1 layer thick so that motility can be accurately assessed in 1 plane and there should be enough room between sperm to assess individual sperm movement. If viewer detects several layers of sperm, sample should be diluted with an isotonic diluent and a less concentrated sample is viewed to improve subjective assessment. One drop of semen from a Pasteur pipette (pipette capacity, ~ 7 ml) is the correct volume to allow for even distribution and movement of sperm under a 22 x 22 mm coverslip placed on a slide.¹¹ Use of a smaller coverslip (18 x 18 mm) may put less pressure on sperm and allow more free movement.

Sperm motility is generally highest at center of coverslip and decreases towards edges. Therefore, viewer should assess those regions across coverslip equator and average motility observed in several viewing fields to increase subjective score accuracy. It is recommended that 2 trained viewers assess slide and average motility value of 2 independent results is recorded. Velocity of movement is also recorded, usually on a scale of 0 - 5 (with 0 being no movement, and 5 being very fast progression).

It is important to differentiate total motility from progressive motility; to do this, individual sperm movement is assessed. If sample is too concentrated, nonmotile sperm may be pushed around by motile ones, leading to an inflated motility score.

Second method for evaluating semen motility is computer-assisted sperm analysis system (CASA), an expensive, automated system that visualizes and digitizes successive sperm images, processes them, and then analyzes information giving precise data regarding motion of individual sperm. When properly calibrated and used with appropriate software parameter settings, a CASA system provides both accurate and precise information on detailed kinematic parameters such as curvilinear velocity, straight line velocity, and amplitude of lateral head displacement.^{12,13} However, machine parameters such as imaging hardware and software, recording quality and camera frame rate can alter motility results.¹⁴ Concentration of sperm, extenders, amount of debris, crystals and immotile dead sperm heads present in sample also alter motility results when using CASA systems.¹³ These issues, compounded by intensive staff training and continuous standardization and machine maintenance, make CASA use challenging in private practice.

Sperm concentration and number

Sperm reservoir depletion decreases sperm concentration,¹⁵ so frequency and interval of prior collections before performing an evaluation should be known. Sperm concentration can be determined manually or using automated methods. Once the concentration is known, total sperm number is calculated by multiplying concentration (# sperm/ml) by total volume (ml) of ejaculate collected. In dog, normal total number of sperm in ejaculate is 300 million - 2 billion.² Sperm production is dependent on grams of testicular tissue; total sperm number is ~ 10 million sperm per pound of body weight. For example, a typical value for a 10-pound dog would be 100 million sperm.

Manual hemocytometer has long been considered the gold standard for precisely counting canine sperm. However, the Nucleocounter[®] SP-100[™] (Chemo-Metec A/S, Allerød, Denmark) discussed below has replaced it in recent years.^{16,17} This manual method is very inexpensive and accurate when performed by trained staff. It is important to use specified cover slip corresponding to hemocytometer and to count both chamber sides. If there is more than 10% discrepancy between sides, chambers should be refilled, equilibrated and recounted. To maintain errors < 5%, it is recommended to count a minimum of 400 sperm per sample.¹⁸ Care must be taken to properly fill chambers and count all sperm heads in designated counting grid only once to improve accuracy.

Differences in sperm concentration between CASA systems and hemocytometer are largely due to the Segre-Silberberg effect, which does not occur when using a hemocytometer due to a deeper sample chamber compared to most disposable slides used in automated systems.¹⁷ Problems with sperm count are similar to assessing motility, sperm count can be inaccurate when low or high concentration semen samples are analyzed. CASA reliability is very dependent on users technical competence.¹⁸⁻²⁰

Another automated method is the Nucleocounter[®] SP-100[™]. Sperm treated with a detergent are aspirated into a cassette lined with propidium iodide, which crosses cell membrane and binds specifically to DNA. Fluorescently labeled sperm are then quantified. Because sperm identification is so specific to DNA, there is no interference from debris, concentration, or extenders.²¹ Pipetting must be done correctly for this machine to provide accurate counts.

Photometric devices such as the Spermacue[®] or Densimeter[®] that depend on quantitative light transmission measurement through a diluted semen sample are easy to use, and are relatively inexpensive. Samples that contain a lot of debris (e.g. leukocytes, bacteria, cytoplasmic droplets, etc.), are extremely concentrated, or very dilute should not be run using photometric devices, as results will be inaccurate.²⁰ Only raw semen can be used in photometric devices; extended semen can only be used if the extender has no optical density (i.e. is clear).

Flow cytometry using detection of light scatter and fluorescence of individual sperm is a very precise and accurate method to determine sperm concentration. However, equipment cost, need for skilled technicians, and involved sample preparation techniques limit this technique to research.

Quality control is critical, irrespective of whatever form of counter is used. When semen is evaluated grossly and microscopically, an estimate of sperm concentration should be performed. Motility samples should be assessed for extraneous cellular debris (epithelial cells, WBC, RBC) that may interfere with accurate counting. If semen is centrifuged, the size of the sperm pellet can be used to estimate total sperm in the ejaculate. If the count calculated by whatever method is used does not match with estimated count based on the above factors, count should be repeated or calculated using another (ideally more accurate) method.

Sperm morphology

Assessment of sperm morphology is a subjective evaluation susceptible to large discrepancies between evaluators. A 79.4% inter-laboratory variability is reported in assessing canine sperm morphology.²² Phase contrast or differential interference contrast microscopy is one technique to view sperm fixed with formol buffered saline as a wet mount, thereby limiting damage done to sperm using staining techniques necessary for light microscopy.^{2,23}

Specific structural defects are known to be associated with male infertility. Staining techniques help visualize sperm defects when viewed under oil immersion, but compared to phase microscopy, the staining preparation technique may contribute to morphologic artifacts.²⁴ Four common staining techniques (conventional, dipping and blotting, direct mixing and ignition) were compared and it was concluded that as long as slides were made carefully according to protocols, percentage of abnormal sperm was constant across all 4 techniques.²⁵

Eosin-nigrosin (Hancock's) stain (available from SFT) is a common stain for sperm morphology. Typically, 1 drop of semen is mixed with 1 drop of stain, spread like a blood smear and allowed to dry. Dark slide background from nigrosin facilitates visualization of sperm head, midpiece and tail. This stain is a "vital" stain, meaning that eosin is able to permeate damaged plasma membranes of dead sperm,

staining them pink, compared to white or clear live sperm.^{2,20} Sometimes there is only partial staining of sperm and staining may not be consistent if there are fat globules in seminal fluid² making it more challenging to clearly designate sperm as alive or dead. Round cells (germ cells, erythrocytes and leukocytes) and acrosomes cannot be easily differentiated.

Giemsa-Wright stain (Diff-Quick TM Baxter Healthcare, Miami FL; Romanowski stain) is a quick, inexpensive, 3-step stain that most clinics use for blood smears. Slide is immersed 5 minutes sequentially in fixative, safranin, and crystal violet, lightly rinsed with water to remove stain and then dried. Artifacts may be minimized by drying the stained smear on a slide warmer set at 37 degrees C or by blowing.² This stain is good for round cells but cannot be used to assess acrosome and cytoplasmic droplets are difficult to observe.

Spermac stain is more labor intensive and costly, however, an excellent stain for evaluating acrosome, equatorial, midpiece and tail regions of sperm.²⁰

Normal percentage of morphologically normal sperm (MNS) for canines should be $\geq 80\%$ ^{6,9} and fertility appears to be affected if there is $< 60\%$ MNS.² A minimum of 100 sperm are evaluated and the abnormal cells classified as: 1) primary (occurring during spermatogenesis) or secondary (occurring during maturation or sample preparation); 2) major (negatively affecting fertility) or minor (not associated with fertility); or 3) compensable (improved fertility by increasing sperm numbers) or non-compensable (fertility not increased by increasing sperm numbers).

Other cell types in semen samples

In addition to sperm, semen samples may contain erythrocytes, leukocytes, epithelial cells and immature germ cells. These are usually noted on the breeding soundness evaluation sheet as numbers of cell type/100 sperm.

Advanced tests

Many specialized stains, assays, and tests are available to evaluate sperm morphology, function, chromatin, membrane integrity, etc. in dogs displaying suboptimal fertility with normal semen evaluation values. One example is the hypoosmotic swelling test, used to evaluate membrane integrity of sperm by assessing tail curling after incubation in hypoosmotic solution.

Conclusion

Routine semen evaluation is an integral part of a canine breeding soundness exam, easy to perform and a valuable tool to evaluate sperm. Results will only be reliable and accurate if equipment is routinely maintained and calibrated. To minimize error, laboratory personnel should undergo training protocols and run standardized procedures with quality control. Perhaps someday we will be able to perform a semen evaluation and, using a new parameter, reliably predict fertilizing potential.

Conflict of interest

There are no conflicts of interest to declare.

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“Boys in a box”- chilled and frozen canine semen shipments

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Abstract

Canine semen used for shipping or cryopreservation should meet minimal semen evaluation standards for concentration, motility and morphology to maximize artificial insemination success. Samples with increased morphological abnormalities may benefit from filtration or gradient centrifugation; however, total numbers will be much lower. Extenders are added to sperm-rich fraction to supply energy source, buffer the environment, decrease bacterial growth and protect sperm membranes during chilling and freezing. Many commercial and homemade extenders are available, along with various shipping box options, to successfully transport semen virtually anywhere in world. Additional cryoprotectants are added to frozen semen extenders to prevent ice crystal formation within sperm. Standardized protocols and routine maintenance of nitrogen tanks, and vapor shipper dewars increase chances of successfully protecting, storing and shipping canine semen.

Keywords: Chilled semen, cryopreservation, semen extenders, canine, dog

Introduction

It has become very common to use chilled and frozen semen for canine artificial insemination, due to convenience and advantages of shipping semen instead of costs and risks of shipping bitches or dogs to be bred, or driving long distances for a natural breeding. Frozen semen can store genetic material of breed lines for later use and also helps expand genetic diversity. Protocols must be followed when shipping or receiving chilled or frozen semen to ensure highest semen quality at insemination.

Stud dog

Goal of breeding is to supply healthy puppies for companionship, service, etc. Therefore, all dogs collected for chilled or frozen semen use should have a normal physical examination, be current on health clearances and vaccinations and be *Brucella canis* negative. A DNA number is required by AKC in order to register puppies born using chilled or frozen semen. This is easily obtained by mailing a cheek swab. To maximize chances for a litter, only semen that meets minimal evaluation parameters in regards to concentration, motility and morphology should be chilled or frozen. For international shipments, collecting veterinarian should check with USDA regarding stud dog mandatory health testing required PRIOR to collecting semen to meet importation requirements for destination country. Current recommendations are available online (www.aphis.usda.gov).

Semen preparation

Presence of first or third fractions of ejaculate may adversely affect semen motility and morphology.¹ These 2 fractions, along with urine, can be removed with supernatant after centrifugation. Regardless, very effort should be made to capture only second (sperm rich) fraction during collection.

Filtration procedures or centrifugation gradients may improve semen quality. With filtration, ejaculate is passed through a filter (e.g. glass wool). Using a centrifugation gradient, sample is carefully pipetted on top of a layer of specialized media in a test tube and centrifuged. Normal sperm move through media and remain on tube bottom as a pellet while media binds abnormal sperm. Some media may aid in blood removal from sample, particularly digested blood, and centrifugation media can also be used to remove urine. These 2 procedures may improve percent morphologically normal sperm; however, drastic reduction in total numbers, especially with glass wool filtration, may preclude shipping or freezing that sample.^{2,3}

Extenders

Canine semen can be shipped at refrigerator temperatures (4 - 5°C) and used within a few days or frozen and stored in liquid nitrogen (-195°C) to be used until years later. Regardless, sperm-rich portion will need to be mixed with an extender, a liquid preparation supplying multiple benefits.

Semen extenders contain energy source (e.g. glucose or fructose⁴) to support motility and contain buffers to help stabilize pH and osmolarity of environment offsetting acid-base changes of metabolism during storage.⁵ They also contain a protein source (e.g. egg yolk or milk) to protect sperm membrane integrity during temperature fluctuations during chilling, freezing and thawing. Percent progressively motile sperm declines gradually with time in extended samples chilled to 4 - 5°C, with slower decline in extenders containing 20% egg yolk compared to skim-milk based extenders.⁵

Commercial preparations have ingredients that are often proprietary and vary with their composition (Camelot Farms, College Station, TX; International Canine Semen Bank, Sandy, OR; Synbiotics, Kansas City, MO; CLONE, Doylestown, PA; and Minitube, Verona, WI). Many formulas available for homemade extenders; however, these have increased chances for more variability between batches.

Preparing and shipping chilled semen

Prior to shipping, inseminating veterinarian should inform collecting veterinarian about type of insemination being done and preferred volume desired. Proper labeling of all plastic or glass tubes containing semen with permanent marker is paramount! The Society for Theriogenology has guidelines on their webpage (www.therio.org) regarding tube identification and paperwork recommendations to ensure correct identification. Records annotating semen evaluation results, extender used, dilution, volume, etc. should be in duplicate, with clinic retaining a copy. An established clinic protocol should be followed for every shipment.⁶

Collected ejaculate is centrifuged (300 - 700 x g for 5 - 6 minutes) if it contains any of first or third fractions, after which supernatant is withdrawn and extender added (at similar temperature as semen). Most commercial extenders recommend a dilution of 1 part semen to 4 parts extender. Usually, 2 - 6 ml for intrauterine or a transcervical insemination and 1 - 10 ml for vaginal inseminations are shipped, depending on bitch size and amount requested by inseminating veterinarian. A small aliquot should be removed and refrigerated until shipped sample is evaluated upon arrival. This saved sample can be used to evaluate semen if it arrives in poor condition at insemination facility to determine if semen did not chill well or if something happened to the box after leaving collection facility (i.e. temperature extremes). After cap tube is secured, tube top is wrapped with Parafilm[®] (Bemis Company, Neenah, WI). Scotch tape, strapping tape, and duct tape should not be used as they do not provide a watertight seal. Plumber's tape, if used, should not have any contact with semen as it may be spermicidal (depending on brand).

To maintain a cool temperature, most commercial semen transport boxes are lined with extruded polystyrene foam and use 1 or 2 plastic liquid bricks that will need to be frozen prior to shipping. To ensure that extended semen does not come into direct contact with frozen brick, tube and frozen brick(s) should be thoroughly wrapped with either several layers of newspaper or some form of packing material, e.g. bubble wrap. Chilled canine semen shipped in Equitainer[®] (Hamilton Research Inc, South Hamilton, MA), a plastic shipping container that is primarily used to ship stallion semen, had highest percent motility 48 hours post-chilling compared to a styrofoam box or a Thermos flask⁷. This container is also approved for multiple uses. A new thermos system called Ship-Mate[®] (My Breeder Supply, Elk City, OK), is an electronic-chilled vacuum flask that uses a battery inside a thermos to maintain temperature at 5°C for duration of transport and can record temperature. Research is needed to verify accuracy.

Chilled semen should be shipped as quickly as possible and maintained between 4 - 5°C to ensure optimal quality upon delivery. Fedex and UPS are both reliable for pickup and overnight deliveries. When shipping on a Friday for a Saturday delivery, be sure to mark appropriate box on shipping label to avoid delivery on Monday. When shipping on Saturday for a Monday delivery, be sure to mark the waybill as an overnight shipment; otherwise, it will be delivered on Tuesday if marked as 2 day shipment.

Receiving a chilled semen shipment

A chilled semen shipment should be opened upon arrival and frozen bricks inspected. If they are warm, semen temperature is most likely too warm and could influence semen quality and bacterial growth. After 48 hours, extended canine semen maintained at 4 - 5°C with antibiotics effectively controlled aerobic bacterial growth, but not when held at room temperature.⁸

A drop of extended semen should be warmed on a 37°C slide warmer and examined for motility, morphology and a general estimate of total numbers to compare to data on shipping form. Any large discrepancies should be reported to collecting veterinarian to allow for changes in future planned shipments, e.g. using a different extender. Stud dogs that have never had semen chilled should have a “chill test” done several weeks prior to shipping where sperm rich fraction is extended using various extenders, refrigerated overnight and motility and morphology verified 24 hours later to determine the extender that maintained best quality overnight.

If insemination cannot be performed immediately, extended semen should be refrigerated and used as soon as possible. Extended chilled semen is best used within 48 hours of collection.⁵ Canine semen tends to tolerate chilling very well and may often maintain excellent motility for many days (sometimes up to 8 - 10) regardless of extender used. You do not have to use a specific 5 or 10 day extender to expect similar results; most semen that ships well in a 5 or 10 day extender will ship just as well in many other extenders. Some of 5 and 10 day extenders seem to promote agglutination of sperm to coverslip upon warming; although this does not appear to affect fertility, it makes it difficult to accurately assess motility.

Frozen semen principles

Frozen semen extenders are very similar in makeup to chilled semen extenders, except most contain glycerol and egg yolk as cryoprotectants to stabilize and protect sperm exposed to the damaging temperatures of liquid nitrogen. During cryopreservation, glycerol, ethylene glycol, or dimethylsulfoxide⁹ permeates through sperm plasma membrane, interacts with water molecules, and inhibits formation of hydrogen bonds necessary for formation of damaging ice crystals. Egg yolk contains lipids lecithin and cephalen⁵ that are too large a molecular weight to cross cell membranes; therefore, ice crystal formation is inhibited by causing cellular dehydration, freezing point depression and increasing media viscosity at lower temperatures.^{9,10}

Semen is slowly cooled to 4 - 5°C and then frozen in either straws or pellets. Prelabeled polyvinylchloride straws, 0.25 or 0.50 milliliters in volume, are filled with cooled semen and an air bubble located centrally in straw. Straws are then sealed and frozen by suspending them over liquid nitrogen vapor and then plunging them directly into liquid nitrogen.⁵ Straws are placed in canes and stored vertically in goblets in liquid nitrogen cryotanks. Pellets are made by placing individual 50 - 100 µl drops of cooled semen into small depressions made in dry ice and then immersing pellets into liquid nitrogen and storing them in pre-labeled cryovials.

Decision to freeze semen in pellets versus straws is personal choice. Pellets are convenient in that the entire vial, usually a calculated amount to breed 1 bitch, is thawed, whereas straws are thawed individually. Pellets take less room to store, as vials are packaged in a box and several boxes fit on a cane; each goblet can only hold ~ 6 - 8 canes of straws. Straws, however, are more accepted internationally. Straws also prevent cross contamination with anything that may be in the liquid nitrogen and make it impossible to combine or mix semen from 2 animals together.

Vapor shipper dewars

Frozen semen is shipped in a “dry shipper” dewar, a vacuum-insulated canister lined with a hydrophobic material that absorbs liquid nitrogen, thereby keeping the frozen semen stable in nitrogen vapor during transport. Liquid nitrogen itself is labeled as a hazardous material by US Department of Transportation and cannot be used for shipment, making it necessary to use a dry shipper to transport semen.¹¹ Canister is housed in an outer heavily padded, plastic container that helps keep the canister upright during transport.

A face shield and protective insulated gloves should be worn when working with liquid nitrogen. Canister will need to be filled, or “charged” 24 - 36 hours prior to a shipment, by slowly adding liquid nitrogen to canister using a funnel until level reaches bottom of the neck. Care must be taken not to spill liquid on vacuum cap as this may cause shrinkage of seal allowing air to leak into vacuum space. Neck plug and lid are replaced and canister is allowed to sit dormant so nitrogen can permeate absorbent. This process is repeated until level of liquid nitrogen no longer falls. Weigh shipper and check manufacturer’s manual as there are suggested full weights to indicate normal absorbent filling¹² and holding times.

Evaporation rate of a vapor shipper is calculated to monitor charging capability. Vapor shipper is charged, liquid nitrogen dumped out and tank weight recorded. Canister is weighed again after sitting dormant for 24 hours and second weight recorded. Evaporative loss per day is calculated by multiplying difference between 2 weights by 0.5606. If this number is > 1.9 (liters/day), vapor shipper should either be replaced or sent to manufacturer to be revacuumed. Vapor shippers should also be weighed prior to shipping and when they return, to confirm that evaporative rate/day is within acceptable limits.

Frozen semen shipment

Just prior to shipping, tank needs to be inverted over an insulated bucket and held until liquid no longer runs out. Canister neck plug and lid should be replaced and canister watched for 1 hour to be certain no condensation or frost collects around top that could indicate a leaking tank. Canes loaded with straws or cryovials are then transferred to goblet inside canister, and neck plug and lid inserted. Lid is then anchored shut with a zip tie. All paperwork (copy of freeze report, instructions for thawing, and return Fedex label) is placed in a manila envelope next to canister, along with any necessary supplies for inseminating veterinarian and outer container is closed and anchored with a zip tie, or left unanchored if canister needs to be sealed by US Department of Agriculture for an international shipment. Like chilled semen, Fedex or UPS are preferred carriers and tracking numbers should be sent to receiving veterinarian.

Receiving frozen semen

When you receive a frozen semen shipment, container should first be inspected for damage. It is then opened and inner canister lid carefully removed watching for a vapor “puff,” confirming that canister is still charged. Contents should be quickly transferred to a bucket of liquid nitrogen, carefully inventoried to verify shipment of correct semen and then transferred into a regular liquid nitrogen storage tank, as shipper stability is unknown. If you do not have any cryostorage tanks and need to hold frozen semen a few days before using, it is best to either add liquid nitrogen to dry shipper daily or arrange for arrival of frozen semen on insemination day. Before returning canister, remove goblet and invert it over cryobucket to ensure that a cryovial or straw did not dislodge from cane during transit.

Closely examine cryovials containing pellets to be sure that vials themselves are not fractured into tiny shards and that semen is still in a round, pelleted form and not a thawed glob on vial bottom. Straws should also be examined for damage such as cracking or extrusion of end plugs. Any signs of tank failure or damage should be immediately reported to vapor shipper facility.

Maintenance of storage canisters

Liquid nitrogen level in storage tanks should be measured weekly using a calibrated cryostick and recorded in a log to monitor evaporation loss prior to topping them off. Any tank evaporating at a faster rate than previously noted should be removed from service.

Conclusion

Canine semen is routinely being shipped everywhere in US as well as internationally, allowing ease of breeding and increasing availability of diverse genetic material. There are many extenders and shipping box options available for chilled semen to preserve semen quality during transport. Frozen semen can be stored indefinitely, allowing access to several genetic lines. A great deal of paperwork and maintenance is involved when dealing with semen shipping and freezing to ensure quality and accuracy.

Hopefully, evidence-based research will provide standardized protocols for canine semen shipping, cryopreservation and insemination in near future to increase chances of producing healthy, normal-sized litters.

Conflict of interest

There are no conflicts of interest to declare.

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Canine nomograph evaluation improves puppy immunization

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Abstract

Immunization failure in puppies by modified live vaccines for canine distemper virus and canine parvovirus can occur due to interference from maternally derived antibody. Quantitative measurement of specific antibody (via half-life degradation analysis; nomograph) is available to determine passive transfer from dam. Nomograph enables vaccination timing and followup titer testing tailored for each litter. Objective was to evaluate effectiveness of this approach and use of a nomograph. Puppies (506 puppies < 1 year) that had nomograph completed for their dam were not different in protection rate compared to vaccinated adults and were proven immune at 15.9 weeks. A cohort of similar puppies at 21.6 weeks that did not have nomograph completed for their dam was more likely to have not responded to vaccination.

Keywords: Canine, nomograph, titer, vaccination, distemper, parvovirus

Introduction

Maternally derived antibody (MDA) interference with vaccination is considered as main cause for “failure to immunize” in puppies < 6 months old.¹⁻⁴ Determining breeding dam antibody titers and applying half life degradation analysis to litter (nomograph) improved timing for canine distemper virus (CDV) vaccination.⁵ This concept was not widely adopted, since very few diagnostic laboratories offered canine vaccinal antibody testing. Beginning in late 1990’s, an alarming increase in adverse reactions to vaccines, most notably feline vaccine associated sarcoma,⁶ triggered veterinary profession to reevaluate annual vaccinations, standard practice at that time, for pet dogs and cats. American Association of Feline Practitioners and later American Animal Hospital Association developed vaccination guidelines for cats and dogs, respectively.^{7,8} World Small Animal Veterinary Association (WSAVA) also published similar vaccination guidelines.⁹ These guidelines described vaccines in 3 categories (core, noncore or not recommended). Core vaccines are able to provide complete protection against widespread diseases that induce high morbidity and mortality. For canine, these vaccines are CDV, canine parvovirus (CPV2), canine adenovirus (CAV1, CAV2), and rabies. Guidelines urged that all dogs receive CDV, CAV1, CAV2 and CPV2 vaccines to provide protection from infection for as long as 9 years.¹⁰⁻¹⁵ As a result, early guidelines suggested administration of core vaccines to adult dogs at not more than 3 year intervals.

Sterile immunity is defined as stage of humoral immune response that “sterilizes” a specific virus, thus totally preventing infection. This applies both to vaccine and disease causing wildtype viruses. Modified live viral vaccines ability to infect and replicate are blocked in face of high antibody titers. This blockage happens whether antibody is produced actively by dog or passively acquired in puppy. Modified live viral vaccine neutralized by antibody provides no benefit to puppy or adult; actively immune dog usually has no increase in titer, whereas a puppy remains immunologically naïve.⁴

Currently, canine vaccinal antibody testing is fast becoming part of standard veterinary care. Multiple laboratories across North America offer quantitative antibody testing to determine immunity against CDV and CPV2, and qualitative, point of care antibody screening tests are also available to clinicians. Improved availability of testing, coupled with an increased interest in appropriate vaccine use, contributed to growth in this area.¹⁶⁻¹⁸

Although vaccine adverse reactions are rare, this risk is not offset by benefit in actively immune dog with titers above sterile immunity thresholds. Most recent version of AAHA canine vaccine guidelines suggest antibody testing helps clinicians balance risks and benefits of vaccination.⁸

Puppies present a unique challenge regarding antibody testing, due to potential for residual maternally derived immunoglobulin and likelihood of immunization failure. Most recent WSAVA

vaccine guidelines recommend antibody testing of puppies at 6 months of age⁹ since all initial levels of MDA will have dissipated. This is also the earliest age that qualitative point of care tests may be applied, according to manufacturer. Testing pups at 24 weeks is a significant improvement over past practice of simply assuming vaccinated pups are immune. Unfortunately, however, testing at 6 months of age still leaves a population of puppies at potential risk of disease, especially during this critical period.

Because a clinician usually does not know potential MDA levels, multiple doses of vaccines are given to puppies over many weeks in an effort to both deliver an effective dose as early in puppy's life as possible and to continue until such time as maternal antibody is believed to have dissipated. In some situations, litters may be vaccinated as frequently as every week, beginning as early as 5 weeks of age, potentially increasing chances of adverse events (e.g. immunosuppression or allergic reaction).

Although a definite link between puppy core vaccination and hypertrophic osteodystrophy was not established,¹⁹ producers of dog breeds at highest risk for hypertrophic osteodystrophy (Weimaraners, Irish Setters, etc.) were among first to request nomograph service through our laboratory. Although initial impetus was to avoid "shot gun" approach of administering multiple doses of vaccine over many weeks, it was soon realized that nomographs also allowed followup quantitative titer testing of puppies much younger than 24 weeks.

Based on our testing, average titers for breeding dams are approximately 1:640 for CPV2 and 1:64 for CDV. However, range of titers was quite large, from < 1:2 (negative) to 1:20,480. Depending on antibody amount transferred at birth, age that a litter had no maternal antibody ranged from day of birth to 22 weeks of age.

Because of possible failure of passive transfer, nomograph should not be used to predict protection against wildtype viruses. Breeders with known elevated risk of parvovirus in their kennels are urged to submit samples collected directly from selected pups at 3 - 4 weeks of age. This approach controls for failure of passive transfer and provides a direct measurement of maternal antibody for half life degradation analysis. Because it can be difficult and stressful to collect serum from small puppies, naïve puppy baseline titer testing is only suggested in the face of high disease risk.

Rather than an evaluation of protection for a litter, nomograph analysis of breeding dam antibody level is intended to be a conservative estimate of duration of maternal antibody interference with modified live viral vaccines. Reported percent transfer estimates of 60 - 70%¹ was confirmed by our laboratory (data not shown). However, because we had transfer rates up to 100% in some excellent colostrum producing dams, nomograph is calculated based on conservative assumption of 100% transfer from dam to litter.

Best timing to collect bitch sera for nomograph analysis is 2 weeks before expected whelping date or 2 weeks after whelping. Active colostrum production time is avoided, as circulating antibody titers are decreased due to sequestration of immunoglobulin G (IgG) in mammary gland. Ideally, nomograph should be completed for each litter in order to generate most accurate interpretation of puppy results followup testing. At minimum, a nomograph should be completed within 1 year before whelping.

Once dam's titer is known and set at 100% transfer to her litter at day 0, half life degradation analysis is applied. Rates of specific antibody decline for canine vary from 9 - 12 days.^{1,5} Rate of 12 days was chosen to provide a more conservative estimate. Standard deviation inherent to assays is graphed by showing titers 1 dilution above and 1 below reported titer.

Variability of MDA titers between pups in a litter is within test variability parameters in majority of litters we tested for prevaccination baseline (complete data not shown). Litters tested include submissions from private breeders concerned about parvovirus in their kennels, as well as purpose bred Beagle litters for selection to vaccine research studies.

Vaccine administration is suggested when degradation analysis estimates indicate first successful immunization.^{1,5} A second dose of vaccine is suggested when interfering MDA is very highly likely to be completely catabolized. These ages often vary between distemper and parvovirus; however, most often CPV2 titers are higher than those against CDV. In these litters, an additional dose of monovalent parvovirus vaccine is suggested at an older age. With high maternal titers against both CDV and CPV2, a dose of combination vaccine at 8 - 9 weeks is suggested as "optional". Although it is prudent to give a

dose of combination core vaccine to puppies before transfer to new homes, stakeholders will know that there is a good chance that this dose may not successfully immunize. Quantitative titer testing is suggested 2 weeks (completion of MDA degradation) after presumed final dose of vaccine.

Materials and methods

Serum samples submitted over a 3 year interval from across US and Canada to companion animal vaccine and immuno diagnostics Service (CAVIDS) laboratory were used. Samples were grouped based on whether nomograph had been completed for the dam or not. Protection rates for sera from these groups were compared against those of a group of sera from > 5,000 individual adult dogs with history of vaccination. For all groups, including adult dogs, only first sample submitted for an individual was included for analysis. Data (date of birth and vaccination history of commercial canine vaccinations) of dogs \leq 1 year were used.

Nomograph followup group included 506 individual puppies, with an average age of 15.9 weeks (range 8.7 - 50.9). This group included 202 distinct litters from 188 dams, with 49 breeds represented. Golden Retriever made up 38.3% of this group, followed by the Labrador Retriever at 11.2% (Table 1).

Table 1. Top 5 breeds' percents in nomograph group

Number of pups	Top 5 breeds - Nomograph Group	Percent total
194	Golden Retriever	38.3
57	Labrador Retriever	11.2
24	Nova Scotia Duck Tolling Retriever	4.7
21	Rottweiler	4.1
21	Soft-Coated Wheaten Terrier	4.1

Group without nomograph included 235 individual puppies at average age of 21.6 weeks (range 7.7 - 49.4). This group was comprised of 90 breeds, including "mixed breed". Labrador Retriever was 18.3% of this group, followed by Golden Retriever at 13.2% (Table 2).

Table 2. Top 5 breeds' percents in without nomograph group

Number of pups	Top 5 breeds - Without nomograph Group	Percent Total
43	Labrador Retriever	18.3
31	Golden Retriever	13.2
24	Irish Setter	10.2
15	Mixed breed	6.4
12	Poodle, standard and toy	5.1

Serology methods included hemagglutination inhibition assay to determine antibody against CPV2 and serum virus neutralization assay to determine antibody against CDV. Both assays are considered "gold standard," directly test antibody function, and provide quantitative endpoint titers. Test sera are doubly diluted in duplicate across a 96 well plate and incubated with a standardized amount of infectious CDV or CPV2. After 1 hour, indicator cells are added to all wells. Plates are examined after further incubation. Endpoint titer is reported as the last dilution at which antibody is able to neutralize viral activity.^{20,21} Threshold of protection determined by challenge of immunity.¹⁰ Both HI and SVN assays detect IgG and IgM simultaneously and are well suited to test initial vaccine responses 2 - 3 weeks after presumed final dose of vaccine.

Data analyses

Chi square was used with significance of $p < 0.05$. Protection rates of both groups of pups were compared against protection rates of a general population of vaccinated adult dogs. Threshold of

protection for adult dogs was set at $\geq 1:8$ (CDV) and $\geq 1:40$ (CPV2). Using this threshold, expected rate of protection is 97% for CDV and 91% for CPV2. To provide a more stringent definition of protection for puppies, and to be able to compare groups, threshold of protection for puppies was set 2 fold higher for both viruses with $\geq 1:32$ for (CDV) and $\geq 1:160$ for CPV2.

Results

Protection rate for the nomograph group was 95.7% (484/506) against CDV (95% CI [95.5, 95.9]) and 90.5% (458/506) against CPV2 (95% CI [90.3, 90.7]). Average age of protected puppies was 15.9 weeks for both viruses. Compared to rates of protection in adult population, p values were 0.0755 (CDV) and 0.7024 (CPV2), indicating no significant differences.

Protection rates in the group without nomograph were lower at 85.5% (201/235) against CDV (95% CI [85.2, 85.8]) and 81.7% (192/235) against CPV2 (95% CI [81.4, 82]). Average age of protected puppies was 21.3 and 22.5 weeks of age for CDV and CPV2, respectively. Compared to expected values in adult dogs, p values were 0.0001 for both viruses, indicating a highly significant proportion of these pups were unprotected. (Figures 1, 2; Table 3, 4)

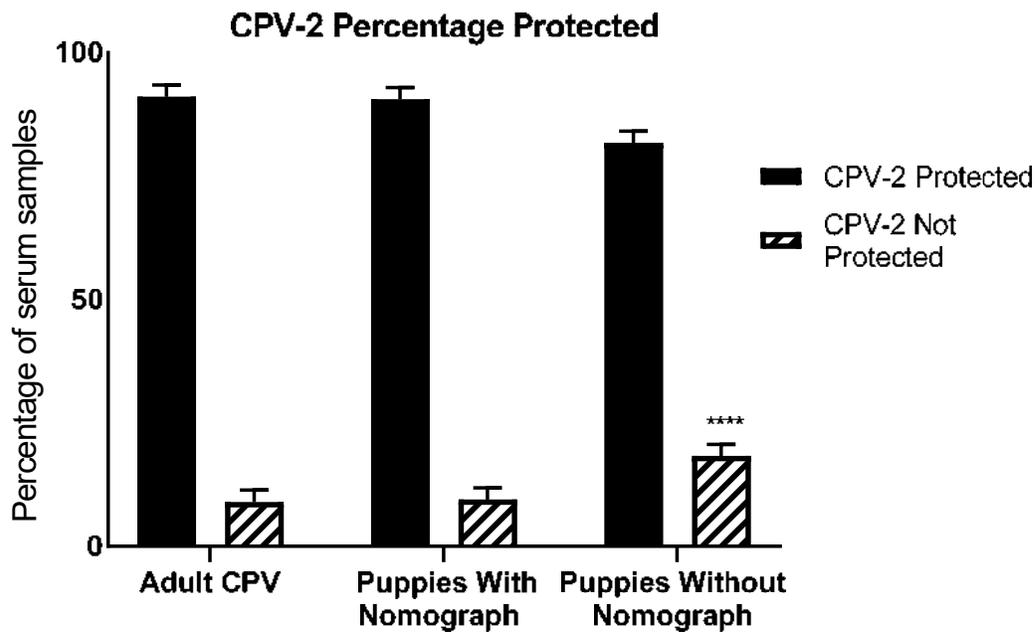


Figure 1

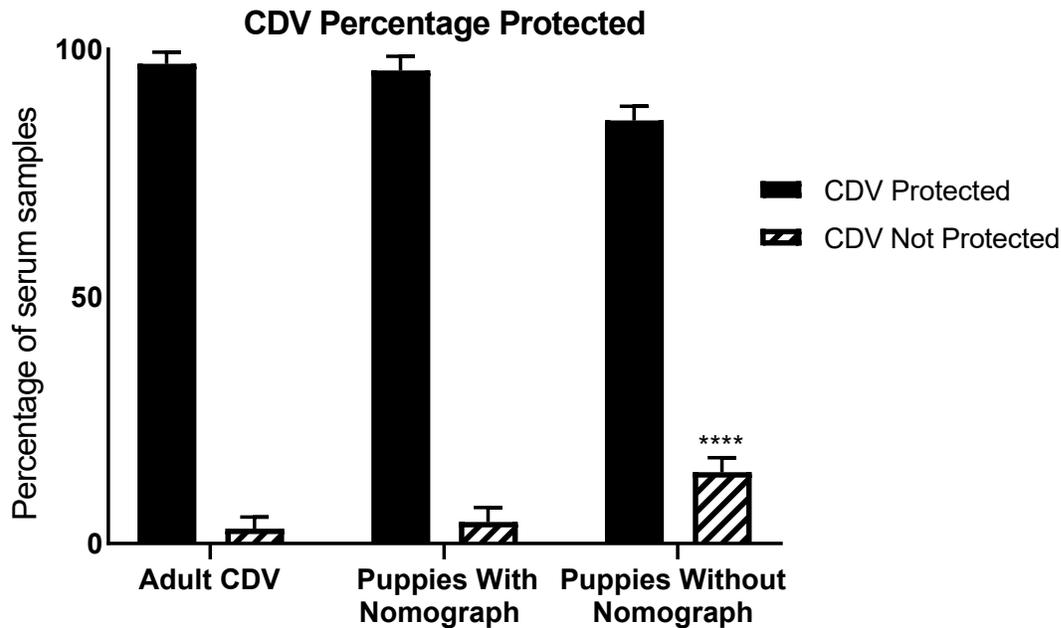


Figure 2

Table 3. Percentage protected/not protected against CPV2 for groups of puppies

CPV-2 ≥160 Group	Percent protected (number)	Average age (weeks)	Percent not protected (number)	Average age (weeks)	P value compared to adults
Nomograph	90.5 (458/506)	15.9	9.5 (48/506)	15.7	0.7024
Without nomograph	81.7 (192/235)	22.5	18.3 (43/235)	17.8	0.0001**

Table 4. Percentage protected/not protected against CDV for groups of puppies

CDV ≥32 Group	Percent Protected (number)	Average age (weeks)	Percent not protected (number)	Average age (weeks)	P value compared to adults
Nomograph	95.6 (484/506)	15.9	4.3 (22/506)	14.3	0.0755
Without nomograph	85.5 (201/235)	21.3	14.5 (34/235)	23.5	0.0001**

Discussion

Maternally derived antibody is only 1 potential factor affecting vaccination failure. Other factors include poor vaccine handling and storage, incorrect administration and genetic issues. However, current study confirmed that MDA had a highly significant impact on vaccinal response in puppies.

Examining role of genetics as a risk factor for poor vaccine response was beyond the scope of this study; however, there were some noteworthy findings. Inclusion of 6.4% “mixed breed” puppies in group without nomograph did not seem to have a significant positive impact on protection rates for this group. Interestingly, in nomograph group, most puppies that failed to respond to vaccine were unique within their litters. In most cases, littermates of nomograph nonresponders responded well (data not shown). One litter of 10 Labrador Retrievers was the exception, with 7 pups that failed to respond to CPV2

vaccine. After a further dose of vaccine, 6 of 7 responded well. Seventh pup required 1 additional dose before eventually having an active response at 20 weeks. Mechanism of non-responsiveness in this litter is unknown, but the phenomenon emphasized importance of titer testing puppies to be certain of immunity. It is also interesting to note that this single litter (out of 202 litters tested) comprised 14.6% of 48 puppies that did not respond to parvovirus in the nomograph followup group.

Although nomograph report suggests tailored vaccination and follow-up testing schedules for each litter, veterinarians and puppy owners are free to interpret and implement nomograph data as they wish. In the current study, compliance was not determined precisely, but the nomograph group was generally in agreement with nomograph-generated schedules (data not shown). One notable exception was the single puppy in the nomograph group tested at 8.7 weeks of age. Although testing at this age was not in accordance with nomograph based suggestions for the litter, this sample was retained in the nomograph group data set. This puppy was negative for CPV2 antibody.

In another interesting finding, the youngest puppy in the group without a nomograph was protected against distemper, but not for parvovirus, at 7.7 weeks of age. This pup's distemper titer was very strong at 1:4,096, clearly an active response to vaccine. For this titer to be attributable to MDA, the dam would have had to have an extremely unlikely CDV antibody titer of 1:65,536 with a 100% transfer rate to her litter. In general, samples from very young pups are not submitted when dam's titers are not known. This is reflected in the smaller size and older average age of the group without nomographs. However, in instances where the puppy has had an adverse reaction to vaccination or other health issue that may complicate further vaccination, quantitative testing and "reverse" degradation analysis can be applied, along with a stringent threshold of protection.

Response to vaccination and protection rates for all groups, regardless of age or nomograph status, was much higher for CDV than for CPV2. Although mechanism responsible is not clearly understood, it possibly stems from the fact that parvovirus has more recently emerged into domestic canine population than distemper virus and has experienced relatively fewer generations of coevolution.

Current study confirmed that nomograph analysis of maternal antibody titers against CDV and CPV2 provides veterinarians and dog breeders with useful information to guide litter vaccination decisions and speed confirmation of immunity. In case of low maternal titers, much peace of mind can be gained by early proof of protection, especially for critical socialization experiences, e.g. puppy kindergarten. In the case of higher maternal titers, vaccination series will be extended, in some cases beyond standard final dose at 16 weeks of age. For all litters and stakeholders, overall outcomes are improved. Dog breeders can provide added value to their puppy buyers by providing information about dam's titers. Veterinarians can make better informed vaccination decisions. Most importantly, more families may be spared emotional and financial impacts of severe morbidity and mortality associated with CDV and CPV2 infections.

Future studies could examine factors that may influence nomograph, such as determining if active colostrum production induces change in circulating antibody titers for bitch; improving understanding of extent of influence of litter size on passive transfer rate; establishing if there are measurable differences in antibody degradation rate due to breed-specific body size, caloric restriction and/or metabolism; characterizing impacts of caesarian section on colostrum production and passive transfer; and determining effect of administration of supplemental antibody products (e.g. fresh frozen plasma) on passive antibody titers in recipient pups. Longer-term collection of titer data of puppies vaccinated according to nomograph as they enter young adulthood and beyond is currently underway.

Conclusion

Current study demonstrated that breeding dams nomograph testing provided important information to improve core immunization outcomes for puppies < 1 year and facilitated earlier followup titer testing for immunity. Regardless of whether nomograph was done for a dam, authors support WSAVA guidelines in urging that all puppies are tested for antibody titers by 6 months to detect failure of immunization during this highest risk period. Methods to detect unprotected puppies are readily available to veterinary practitioner, either through point of care screening tests at 24 weeks, or

quantitative testing earlier when maternal titers are known. Best medical practice should support applying this standard. Presumption that vaccination is equivalent to immunization may lead to unfortunate, but avoidable surprises.

Acknowledgement

Dr. James Baker, originator of canine nomograph in 1958, richly deserves appreciation. Authors are grateful to Avidog International, LLC and their dog breeder members who submitted a large proportion of sera for nomograph analysis.

Conflict of Interest

Authors are employed by CAVIDS laboratory offers (fee for service) serology testing, including nomograph and nomograph follow-up testing of puppies. Authors are grateful to CAVIDS laboratory clients for supporting this entire study by their fees.

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Unnatural selection? Considerations in canine and feline genetic counseling

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Abstract

Molecular genetics and growing availability of molecular technologies are expanding exponentially, pushing boundaries of modern medicine. These bring continued challenges for veterinary genetic counseling which often falls behind human counterparts. There is an increasing need for veterinarians to not only understand this technology, but also to counsel clients to use it appropriately and be an informed voice in current political and socioeconomic debates. A basic understanding is important for veterinarians to use available technologies to control genetic diseases while maintaining genetic diversity in population. Veterinarians, particularly reproductive veterinarians, are in a unique position to bridge the knowledge gap between advancements in genetic science and clinical applications in small dog and cat breeding operations. Veterinarians can help breeders utilize tools such as estimated breeding values, inbreeding or heterozygosity estimates and mutation based genetic disease tests optimally in their breeding programs. There is a critical need to understand that mistakes of past should be replaced by exciting technological advances of future to implement breeding strategies that will more effectively reach small animal breeder's goals of producing dogs and cats that not only are what is desired for appearance, temperament, and workability, but also live long and healthy lives.

Keywords: Genetic counseling, genetic testing, estimated breeding values, population diversity, inbreeding coefficient, heterozygosity

Introduction

With recent advances in molecular genetics and growing availability of molecular technology, we witnessed emergence of genomics into our everyday lives. As we still struggle with bioethics, this technology is expanding at an exponential pace. We live not only in a world where we are constantly pushing boundaries of modern medicine, but also where this technology is readily available, even to public. This brings continued challenges for veterinary profession, which has often fallen behind human counterparts in veterinary genetic counseling. There is an increasing need for veterinarians to not only understand this technology, but to counsel our clients regarding implementation of this technology, as well as to have a voice in current political and socioeconomic debates.

Currently, there are > 700 known hereditary diseases in dogs and ~ half as many in cats, with a growing number of new diseases reported every year.¹ This led to development and growing need for incorporation of clinical genetics into veterinary practice, with small animal practitioners having an ever growing and vital role in both genetic counseling and detection of potentially new genetic diseases.

Hereditary disease is caused by a DNA mutation that can be passed from parent to offspring, whereas a congenital disease is present at birth. With this distinction, congenital diseases can be genetic, but not all congenital diseases are genetic.² For example, an autosomal recessive gene mutation in Portuguese water dogs results in early-age dilated cardiomyopathy and sudden death (Portuguese water dog juvenile dilated cardiomyopathy); however, perinatal infection with parvovirus can result in myocarditis with resultant myocardial damage, heart failure, and sudden death in young puppies as well.^{3,4} Onset of clinical signs can vary for hereditary diseases. Some hereditary diseases may be apparent at early ages where most common presentations are embryonic/fetal death, stillbirth, or fading puppies/kittens. Unfortunately, many are undiagnosed as breeders and veterinarians may not pursue additional diagnostics in these prenatal and neonatal cases. Traditionally, hereditary disorder clinical signs may not be recognized until after weaning, as musculoskeletal, ocular, digestive and other anomalies may not be readily identifiable during neonatal phase of development. Some diseases may have a much later onset. One example is progressive retinal atrophy; affected animals suffer from retinal atrophy that leads to eventual blindness, with clinical signs rarely apparent before 3 - 5 years of age.⁵

Number and variety of genetic diseases is extremely large and many are very rare, with new diseases recognized at an exponential rate.⁶ As such, it is important for a practicing veterinarian to consult reference sources (listed in Table) to obtain knowledge about a known genetic disorder, breed distributions, and distinguishing characteristics regarding diagnosis, treatment, and control. When previously undefined disorder is encountered, evidence of its genetic etiology should be ruled out.⁷

- Does the disorder occur in a greater frequency within a line or breed than in general population?
- Is the disease more common in animals with a higher degree of inbreeding (you need more than a typical 3 - 5 generation pedigree to reveal a more accurate degree of inbreeding)?
- Does the disease have a characteristic age of onset and clinical course, especially when seen in young animals?
- Is the same syndrome found in another species and is it known to be genetic?
- Is there a specific phenotypic defect or syndrome that is associated with a specific chromosomal abnormality?
- Can the disease process be related to a molecular defect such as a defect in an enzyme pathway, structural protein, or molecular receptor?

It is noteworthy that genetic diseases are not limited to purebred dogs and cats. Although mixed-breed dogs generally have a lower degree of inbreeding, many populations (e.g. local stray cats) may have a higher than expected degree of inbreeding. In general, autosomal recessive diseases are more likely to be expressed when there is a higher degree of inbreeding. However, dominant disease and polygenetic diseases may be just as likely in mixed breed populations as they are in more inbred populations, depending upon the disease and the population.²

Diagnostic approach to identifying genetic disorders

Clinical approach to identify genetic disease begins with a thorough history and physical examination. Additional queries regarding littermates and relatives as well as in some cases, a population medicine approach when dealing with kennels and catteries, will assist with collection of infectious disease, toxin, nutritional, and other important data to be considered in investigation of new diseases. Diagnostic tests generally are required to further support a genetic disorder in a diseased animal.⁸ For example, radiology and other imaging techniques may reveal skeletal malformations, echocardiogram may reveal cardiac anomalies and ophthalmologic examination may further define an inherited eye disease. Routine tests such as a complete blood cell count, chemistry screen and urinalysis may suggest specific hematologic or metabolic disorders and may help rule out many acquired disorders. Based on these findings, additional clinical function testing may more clearly define a gastrointestinal, hepatic, renal, or endocrine problem.^{10,11} Histopathology of a tissue biopsy or a necropsy evaluation from an affected animal are often required for a complete evaluation and definitive diagnosis for animals with a genetic defect. The latter is particularly important when faced with a fading neonatal puppy or kitten, as this may give information vital to surviving littermates as well as future planned breeding; however, this important diagnostic tool is often underused.

Few laboratories provide special diagnostic tests that allow for investigation into a possible inborn error of metabolism (Table). Inborn errors of metabolism include biochemical disorders due to a genetic defect in structure and/or function of a protein or receptor. For example, a deficiency in enzyme β - glucuronidase resulting in the lysosomal storage disorder Mucopolysaccharidosis VII has been reported in German shepherd dogs as well as a mixed-breed dog.¹⁰ Most useful specimen to detect biochemical derangements is urine, as abnormal metabolites are filtered but not resorbed by kidneys.

Table. Some useful websites relating to canine and feline genetic diseases

Listings of available tests and testing center information:

- <http://www.akcchf.org/>
- <https://embarkvet.com/>
- <https://www.optimal-selection.com>
- <https://www.betterbred.com/>
- <http://research.vet.upenn.edu/Default.aspx?alias=research.vet.upenn.edu/penngen>
- <http://www.vmdb.org/cerf.html>
- <http://www.vetgen.com/>
- <http://www.vgl.ucdavis.edu/>
- <http://www.vetdnacenter.com/>
- <http://www.caninegeneticdiseases.net/>
- <http://www.healthgene.com/>
- <http://www.labradorcnm.com/>
- <http://www.vdl.umn.edu/>
- <http://www.vetmed.wsu.edu/deptsVCGL/>
- <http://www.aht.org.uk/genetics.html>
- <http://vetmed.tamu.edu/labs/cytogenics-genomics>
- http://www.babs.unsw.edu.au/canine_genetics_facility.php
- http://www.medigenomix.de/zuechterservice_hund.html
- <http://www.catgenes.org/>
- <http://www.dogenes.com/>
- <http://www.animalsdna.com/>

Databases and recommendations for health screening:

- <http://omima.angis.org.au/>
- <http://sydney.edu.au/vetscience/lida/>
- <http://ic.upei.ca/cidd/>
- <http://www.vet.cam.ac.uk/idid/>
- <http://www.caninehealthinfo.org/>
- <http://www.gdcinstitute.org/>
- <http://www.rvc.ac.uk/VEctAR/>

Metabolic screening laboratory:

- <http://research.vet.upenn.edu/Default.aspx?alias=research.vet.upenn.edu/penngen>

Karyotyping/Cytogenetic services:

- <http://vetmed.tamu.edu/labs/cytogenics-genomics/karyotyping>
- <http://www.vgl.ucdavis.edu/services/index.php>
- <http://www.vet.upenn.edu/RyanVHUPforSmallAnimalPatients/SpecialtyCareServices/MedicalGenetics/ResearchFacilities/CytogenicsLab/tabid/708/Default.aspx>

Selected parentage testing services:

- <http://www.vgl.ucdavis.edu/services/index.php>
- <http://www.vetgen.com/canine-profiling-parentage.html>
- <http://www.vetdnacenter.com/canine-parentage-test.html>
- <http://www.dnares.in/canine-veterinary-genetic-dna-parentage-testing-kits-laboratory.php>
- <http://www.uq.edu.au/vetschool/agl>
- <https://www.pawprintgenetics.com/parentage/product-select/>
- <https://dnaproofofparentage.com/>
- <https://www.happydogdna.com/proof-parentage/>
- <https://www.homednadirect.com/animal-dna-tests/>

Selected DNA storage services:

- <https://www.ofa.org/about/dna-repository>
- http://www.animalsdna.com/www.animalsdna.com/web/page/canine/dna_banking/index.html
- <https://www.vetgen.com/services-other.html>
- <https://www.antagene.com/en/antagene/dna-storage-service>

In breeding/Heterozygosity evaluations

- <https://embarkvet.com/>
- <https://www.optimal-selection.com>
- <https://www.betterbred.com/>

Once identified, defect can be further investigated with more specific protein assays. Section of Medical Genetics at University of Pennsylvania School of Veterinary Medicine is one of few places that perform such tests to diagnose as well as to discover novel hereditary disorders www.vet.upenn.edu/pennngen.⁹

In addition, few laboratories offer cytogenetic studies to evaluate for potential abnormalities in chromosomes (Table). Any cell capable of dividing can be used for this purpose; however, most commonly, blood lymphocytes or skin fibroblasts are used. For lymphocyte culture, blood is collected into sodium heparin, cultured in media and stimulated to divide. Cells are arrested in mitosis during metaphase where chromosomes are compacted. Chromosomes can then be stained to yield a typical banding pattern of chromosomes used in traditional karyotyping, or fluorescent probes can be used in a technique known as fluorescence *in situ* hybridization.

A thorough investigation into the family history of a patient with a suspected genetic disease is also important to determine a potential mode of inheritance. Knowing how a disease is passed from generation to generation is critical to plan a breeding program to manage genetic diseases, as well as starting investigation into a genetic cause of a new disease presentation. Inheritance patterns reported in veterinary medicine include autosomal recessive, autosomal dominant, X-linked recessive, X-linked dominant and complex (polygenetic) diseases.² Recessive diseases account for a majority of diseases for which a known inheritance pattern and a genetic defect are not identified.¹² However, with continued advances in molecular biology and technology, this is also becoming true for complex (polygenetic) disorders.

Types of genetic disorders

Autosomal recessive diseases are identified most commonly at presentation of affected animals with both sexes equally represented born to clinically normal parents. Typically, clinically normal parents have a common ancestor. These animals that are phenotypically normal are referred to as carriers (heterozygous for the disease-causing allele).² Common theories for increased prevalence of expression of autosomal diseases in purebred dog and cat populations include higher degree of inbreeding related to popular sire effects, selective inbreeding, and bottlenecks in their populations.

Autosomal dominant diseases are often seen with an affected individual produced from at least one affected parent, since carrying either one (heterozygous) or both (homozygous) copies of mutant allele will result in disease. However, not uncommonly, new mutations can occur which result in an affected animal that is produced by 2 clinically normal parents.² In some cases, diseases are referred to as being incompletely dominant. Traditionally, incomplete dominance occurs when expression of disease with a heterozygous genotype (one copy of the mutant allele) is an intermediate or has variable disease expression. In these cases, parents with the disease-causing allele may not exhibit any clinical signs and appear normal, yet they may pass that disease causing allele to their offspring. Some theorize that interactions with other modifying genes and in some cases, environment, affect disease expression and severity, making some believe that a proportion of these incompletely dominant diseases may have inheritance patterns more similar to complex modes of inheritance.

X-linked recessive diseases are distinguished mainly by males being predominantly affected. Females are far less likely to be affected based on the presence of 2 X chromosomes and requirement for an affected male to survive long enough to reproduce with a carrier female in order to produce an affected female offspring.⁸ First canine mutation discovered was X-linked recessive disease, Hemophilia B.¹³ X-linked dominant diseases are extremely rare, e.g. X-linked Alport syndrome in Samoyed dogs.¹⁴

Y-linked disorders are caused by Y chromosome mutations. Since males inherit a Y chromosome from their fathers, every son of an affected father will be affected. However, since females only inherit an X chromosome from fathers, female offspring of affected fathers are always normal with Y-linked disorders. Since Y chromosome is relatively small and contains very few genes, there are relatively few Y-linked disorders and none reported in small animals. Another rare mode of inheritance in veterinary medicine is mitochondrial inheritance. This type of inheritance, also known as maternal inheritance,

applies to mutations of genes in mitochondrial DNA. Since only oocytes contribute mitochondria to the developing embryo, only mothers can pass on mitochondrial conditions to their offspring.⁸

Complex disorders are more difficult to identify as they result from combination of effects of multiple genes (polygenetic) as well as environmental influences that result in an expressed phenotype. Although complex disorders often cluster in breed or family lines, they do not have a clear-cut pattern of inheritance as seen with single-gene disorders.² This non-Mendelian inheritance pattern, as is often used to describe complex disorders, makes it difficult to determine an animal's risk of inheriting or passing on these diseases. Complex disorders are also more difficult to study and identify all factors leading to expression of disease. However, common veterinary diseases are increasingly recognized as having a genetic, heritable component. In fact, some common diseases recognized in veterinary medicine meet these criteria, including hip dysplasia, hypothyroidism, cancer and atopy.¹⁵ Some argue that everything has a genetic component and it is how these genes are expressed by a multitude of factors that determines rate of disease. With increasing knowledge of gene involvement in disease, clinical veterinary genetics becomes increasingly important in diagnosis, management and prevention of disease in our patients.

Identification of unknown genetic diseases: genetic tests advantages and limitations

Veterinarians are part of an important team involved in identification and control of genetic diseases. Breeders, pet owners, primary care veterinarians, veterinary specialists, veterinary researchers, genetic databases and research funding institutions comprise vital pieces of this team. When a new genetic disease is suspected, team members need to work together to compile information and resources required to determine gene defect(s) involved. Sometimes this is not straightforward and easy. In general, researchers often modify and combine multiple techniques in uncovering a genetic mutation process, most common being genome-wide association study and candidate gene approach.

Genome-wide association studies compare DNA of 2 groups of participants: affected animals and similar animals without disease (normal controls). DNA is collected from these individuals and gene chips along with computer technology are used to read millions of DNA sequences. However, rather than reading entire DNA sequence, single nucleotide polymorphisms (SNPs) are used. SNPs are variations in a single nucleotide of a DNA sequence, used as markers for evidence of DNA variation. Different variations are then identified along with their association with different traits. If genetic variations are more frequent in diseased animals as compared to normal controls, variations are considered associated with the disease. Associated genetic variations are then considered linked-markers to the region of the genome where the disease-causing problem is likely to reside. Most SNP variations associated with disease are not in DNA region that codes for a protein. Instead, they are usually in large noncoding regions on the chromosome between genes that are edited out of the DNA sequence when proteins are processed. However, once these markers are linked to a disease, further molecular techniques can be utilized to narrow down the region and sequence potential genes, thus identifying mutations.¹⁶

Another method utilized in genetic mutation investigation is candidate gene approach. This approach requires researchers to investigate validity of an educated guess about genetic basis of a disorder, as opposed to genome-wide association studies that are predicated on an unbiased search of entire genome without preconceptions about role of a certain gene. Similar to genome-wide association studies, candidate gene approach involves comparison of affected individuals with normal controls; however, since 1 gene is the focus, large populations are not required for an association with disease to be detected. Major difficulty with this approach is that to choose a potential candidate gene, researchers must already have an understanding of disease pathophysiology and potential genes that may influence the mechanism of that specific disease, e.g. gene mutation known to cause same disease in another species.^{17,18}

Often, linkage to a disease is known before mutation is identified. Linked marker testing can then be utilized to assist breeders in breeding choices before a mutation-based test is established. It is important for veterinarians and breeders to understand advantages and limitations for a particular genetic test to achieve their goals of controlling genetic diseases while maintaining genetic diversity in population as a whole. Several types of inherited disease screening and genetic tests are described in veterinary

medicine, including phenotypic testing, linked-marker testing and mutation-based tests. In short, not all genetic tests are created equally and understanding various types of tests along with mode of inheritance of a disease is vital to use tests appropriately. For example, linked marker testing may have 2 potentials for errors. First error can occur from a recombination event where the marker is no longer linked to mutant allele, resulting in either a false positive or false negative test.² In general, closer the marker is to mutant allele, more likely they will remain together, or linked, and less likely recombination will result in their separation. Another error occurs if the marker is not linked to mutant allele, but is present in a high enough frequency in the population that it may initially appear linked, resulting in a false positive test.² Although caution must be used when interpreting test results, it is important to recognize that a linked marker test is extremely useful when dealing with a disease for which the mutation is not yet known.

Once a gene mutation is identified, it is important to note that these mutations are very specific. Small animals within same or a closely related breed may likely have same disease-causing mutation for a particular disease. However, small animals of other breeds, particularly unrelated breeds, with same disorder, may also have different mutations that may not be detected with a mutation-based test.⁸ There may also be > 1 genetic mutation within a breed that may result in similar clinical signs and in these cases, all mutations need to be evaluated.

DNA tests have several advantages. Test can be performed at any age and long before clinical signs become apparent, detecting affected, normal and carrier animals. DNA can be extracted from any nucleated cell, such as white blood cells, cheek cells, hair follicles, semen and even formalinized tissue. Cheek swabs should be used very cautiously or avoided in nursing animals due to potential contamination of oral cavity with maternal nucleated cells.⁸ Since DNA is very stable and small quantities are required, it can be banked for long-term storage and utilized in future genetic studies. There are several veterinary DNA storage facilities for this purpose. Key factor in usefulness of DNA for future studies is determined by complete and thorough records on that animal. An animal suffering from an inherited disease needs to have an accurate diagnosis of the cause of that disease in order to prevent false associations when utilizing that animal's DNA for a potential gene mutation study. For example, a cat with suspected liver disease due to amyloidosis needs to have histopathological confirmation of that disorder, or there is a risk that an animal with hepatic adenocarcinoma may confuse and invalidate future genetic studies.

Although identifying genes causing disease is of valuable assistance, that alone is insufficient. Understanding how to implement testing and educating clients to responsible use of these technologies is vital. So, knowledge of molecular genetics and current biotechnology is only 1 piece of the puzzle.

Genetic diversity

Development of pedigree animals are due to selection for a particular set of physical and/or behavioral characteristics. This can be considered both a “blessing and a curse”, because the result has been obtained from inbreeding within close family lines resulting in both preservation of desired features as well as undesirable genetic traits becoming fixed within breeds.^{19,24} Closed populations, such as purebred animals, with high levels of selection pressure, suffer from a loss of genetic diversity. For example, in dogs, 2 major events resulted in a significant loss in genetic diversity which only continues in these closed populations. These 2 major events include domestication and development of breeds. In managed populations, genetic diversity can be maintained by careful selection in order to maximize optimal contributions of each breeding animal.^{20,26} However, in most purebred organizations, e.g. dog breed clubs, there is no single organization to assist with directing breeding strategies.²⁴ This is why veterinary genetic counseling is so vital to the future of dog breeding. Individual breeders should have insight into population genetics and methods to preserve as much genetic diversity in these closed breeds as possible.²³ Veterinarians offer a great way to improve that insight and bridge the knowledge gap.

Evaluating genetic diversity

One of the oldest observations of population genetics is that inbreeding reduces fitness in offspring compared to offspring of unrelated individuals. Consequently, most human populations prohibit marriage of related individuals and many animal breeders discuss measures of inbreeding. Inbred

individuals have a lower degree of genome wide diversity or heterozygosity because a fraction of their genome is identical by descent inherited by a common ancestor. All measures of inbreeding attempt to predict the proportion of the genome that is identical by descent.^{21,22} Classical measure of this is the pedigree inbreeding coefficient.^{28,29} Calculated inbreeding coefficient predicts proportion of the genome that is inherited by descent due to known common ancestor of parents and the assumption that the pedigree founders are unrelated and not inbred. While traditionally, the coefficient of inbreeding has been considered the best measure for an individual's inbreeding, it has been suggested to be imprecise due to several factors, including inability to account for inbreeding caused by distant ancestors not included in the pedigree. With development of molecular technologies, genetic evaluations of inbreeding have been replacing traditional probability calculations.²⁷

Several techniques have been described to evaluate genetic diversity being reported as an evaluation of heterozygosity rather than the traditionally reported inbreeding coefficient designed by probability calculations of pedigrees.^{25,26} There are 3 main companies offering genetic testing services that includes an evaluation of heterozygosity. These companies are utilizing either short tandem repeats (STRs) or single nucleotide polymorphisms (SNPs).

A short tandem repeat (STR) in DNA occurs when a pattern of 2 or more nucleotides is repeated and repeated sequences are directly adjacent to each other. An STR is also known as a microsatellite. Pattern can range from 2 - 6 base pairs and is typically in a noncoding region of DNA. As noted above, single nucleotide polymorphisms (SNPs) are genome regions in which 2 strains differ by a single base pair. Both methods of genome scanning have application-dependent advantages and disadvantages.

Microsatellites can arise through replication slippage, unequal crossing over, or mutations extending or interrupting a series of repeats, whereas SNPs generally arise via point mutations. As a result, new microsatellite variations arise more frequently than new SNP variations. However, the absolute number of SNP differences is about a thousand-fold higher than microsatellite differences. Thus, SNP and microsatellite analyses can provide complementary information, with each is better suited for some tasks than others.²⁸

Microsatellites have been the genetic markers of choice for > 2 decades. They are informative and interspersed throughout the entire genome. However, microsatellites can be more time consuming for trained personnel to analyze, even with appropriate software or automated methods. Recent advances in high-throughput DNA sequencing, computer software and bioinformatics have increased popularity of SNPs. They have promising advantages, including greater abundance, genetic stability, simpler nomenclature and suitability to automated analysis and data interpretation. Furthermore, SNPs have been used in discovery of quantitative trait loci (QTL), the association of phenotypic traits and genetic markers allowing for evaluation of specific productive traits and identification of individuals and breeds.²³

Consolidation of tests: current trends

Currently, there is a move toward consolidation of many available genetic tests from small businesses and universities to larger companies offering analysis of genetic diversity (based on SNPs or microsatellites to evaluate in-breeding). Sudden availability of large amounts of genetic data for breeders is frequently misinterpreted and is an opportunity for veterinarians to bridge the knowledge gap. Some of these companies include Embark, Optimal Selection and BetterBred. Optimal Selection currently offers similar testing for cats. Embark and Optimal Selection are utilizing SNPs, whereas BetterBred, which is partnered with the University of California Davis Veterinary Genetics Laboratory, utilizes microsatellites and is available only for certain breeds. In addition to offering tools for evaluating in-breeding of individual dogs, both Optimal Selection and Embark have begun offering a "match making tool" that compares genetic diversity information between individuals of a proposed mating.

Estimated breeding values

Currently, the vast majority of genetic tests available are for single-gene disorders. Many dog and cat breeders continue to rely solely on phenotypic data when dealing with complex disorders such as hip dysplasia. They are mostly screening prospective breeding animals and selecting breeding pairs with this

limited information; their goal is to reduce disease incidence, but the approach has flaws and progress is slow. In this area, we can learn more from large-animal producers who have utilized estimated breeding values (EBV) for decades to manage diseases with more complex inheritance and improve production parameters. Estimated breeding values (EBVs) are the value of an individual as a genetic parent. They utilize all that is known about a trait, in an effort to predict the potential that the trait will be expressed in the offspring. The more that is known about the trait, including the environmental conditions associated with individuals as well as heritability percentage, the greater accuracy.³⁰ Large-animal producers have used EBVs to do everything from increase volume, butterfat and protein in milk to improve production of lean meat from beef animals, to increase feed efficiency and improve reproduction.³⁰

In US beef industry, various breed associations are responsible for calculating EBV's. Most associations, however, contract with a university that specializes in advancing technology for doing the (inherently complex) EBV calculations.³⁰ Major universities where contract computing is done include Iowa State University, Cornell University, Colorado State University and University of Georgia. Computing EBV's for dairy industry is performed by Animal Improvement Programs Laboratory, Beltsville Agricultural Research Center, Agriculture Research Service, US Department of Agriculture where staff work year-round to improve and implement process of calculating EBV's for dairy cattle.³⁰

Estimated breeding values change, as data used to calculate them is constantly evolving. Understanding their use in breeding programs for those unfamiliar with genetics can be overwhelming. For the large-animal industry, the Cooperative Extension Service was very successful in educating farmers on the use of EBVs. One of the most successful tools for teaching beef cattle breeders how to use EBV's was the computer cow game.³⁰ This created real-world scenarios where producers would make breeding choices based on EBVs. Typical of the real-world, a certain percentage of cows in every herd would fail to conceive, calves would die and other calamities would strike. Out of all this mayhem and information, however, emerged new calves with new records that became the basis for a new round of EBV's.³⁰ Selection decisions could be made and the process repeated. Cattle breeders enjoyed competing with each other to see who could produce the greatest genetic improvement in their herd in 5 generations of selection. Such a tool was suggested by prominent canine geneticist, Dr. Elton Leighton, specifically written for dog breeding and implemented online, could be used to teach dog breeders about EBV's.³⁰

Dr. Leighton and the team at the Seeing Eye, have been utilizing estimated breeding values for years to improve genetic selection for dogs to succeed in their program as well as to improve dogs' health, in particular, hip health. They continue to publish successful demonstrations of their research showing how dog breeders can incorporate these techniques to improve selection for these more complex inherited diseases.³¹

The task of calculating EBV's is generally complex and relies on diverse sharing of a large amounts of data, with accuracy of the EBV's based on accuracy of information reported, or lack thereof. To be successfully implemented with dogs, groups of dog breeders, e.g. national parent clubs or the American Kennel Club, need to decide that routinely computing EBV's is an important task to be undertaken. Any of these dog breeder organizations can turn to U.S. beef or dairy industries or Seeing Eye for examples of how to organize data collection and calculation processes. Recent advances in computing algorithms and computer software to implement these algorithms now facilitate simultaneously calculating EBV's on several traits measured on thousands of dogs.³⁰

Conclusion

With growing advancements in molecular genetics and genetic tests being developed at an exponential rate, it is important for veterinarians and breeders to have a basic understanding of how to utilize these techniques in order to control genetic diseases while maintaining genetic diversity of the population as a whole. Emergence of genomics into clinical veterinary practice has led to the need for development of a team-based approach to control and identify genetic disorders in small animals.⁸ Hard work and cooperation among breeders, pet owners, primary-care veterinarians, veterinary specialists, veterinary researchers, genetic databases and funding institutions has resulted in development of nearly 200 genetic tests.⁶ This research has not only benefited the lives of our small animal patients and their

families, but has also increased understanding of genetic diseases in other species, including humans. Going forward, we all need to continue our vital roles in this process so that we can further unlock mysteries behind some very common diseases in veterinary medicine. Veterinarians, particularly reproductive veterinarians, are in a unique position to bridge the knowledge gap between advancements in genetic science and clinical application in small dog and cat breeding operations. We can assist breeders in understanding current genetic status of their own breed so they can work to preserve population size of their breed. We can help breeders utilize tools such as estimated breeding values, inbreeding or heterozygosity estimates, and mutation-based genetic disease tests optimally in their breeding programs. We can help them to understand that the mistakes of past should be replaced by exciting technological advances of future to implement breeding strategies that will more effectively reach their goal of producing dogs and cats that not only are what is desired for appearance, temperament and workability, but also live long, healthy lives.

Conflict of interest

There are no conflicts of interest to declare.

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Pregnancy analytics app adds value to pregnancy diagnosis in beef herds

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Abstract

Determining pregnancy status of beef cattle is an important service both as a major source of income for veterinary practices and a valuable information resource for cow-calf producers. However, value of pregnancy status information should be enhanced to give veterinarians a competitive advantage against alternate methods for identifying nonpregnant cows. Obtaining fetal age estimates is vital to create charts to evaluate and display pregnancy percentages by 21 day periods. Evaluating breeding seasons by 21 day periods and by animal age and/or other management groups is useful to practitioners investigating herds with reproduction shortfalls by identifying specific periods within breeding seasons when cows did not become pregnant. In addition, easy-to-read charts can enhance communication between veterinarian and beef producer by illustrating results of fetal aging. Once fetal age data are collected and organized for analyses, conclusions or further questions will present themselves. Nutrition, genetics, animal husbandry, male and female reproductive soundness and health all affect distribution of pregnancy within a herd. By combining transrectal uterine examination to determine pregnancy status with chute-side analysis and graphing of information, veterinarians can readily identify strengths and weaknesses within herd management.

Keywords: Beef cattle, pregnancy diagnosis, histogram

Introduction

Determining pregnancy status of beef cattle is an important veterinary service. For veterinary practices, it is a major source of income. For cow/calf clients it is a cost-effective source of valuable information. However, to increase value of this service and for veterinarians to have competitive advantage compared to other persons and alternate methods, veterinarians should gain additional information from pregnancy status determination and use key metrics to optimize ranch productivity.

Beef cow reproduction is limited by 2 key factors: first, a relatively long infertile interval after calving and second, only 60 - 70% of successful matings of fertile cattle results in a viable pregnancy. Approximately 30 - 40% of fertile matings result in either failure of fertilization or death of the early embryo, but in most situations, the mated, but non-pregnant cow will express estrus and ovulate a fertile oocyte about 21 days after her last ovulation and will have another 60 - 70% probability of conceiving and maintaining a pregnancy. Cows with 3 opportunities to be bred (each with a 60 - 70% probability of a successful pregnancy) have ~ 95% probability of being pregnant at mid-pregnancy examination. If nearly all cows in a herd calved early enough so that they resume fertile cycles by the 21st day of the next breeding season and bulls are fertile and able to successfully breed, the ideal pregnancy pattern will be ~ 60 - 65% pregnant in first 21 days of breeding season, 85 - 90% pregnant by 42nd day of breeding and ~ 95% pregnant after 63 days of breeding (Figure 1).

BCI pregnancy analytics app: gathering pregnancy data chute-side

Beef Cattle Institute developed a pregnancy analytics app to assist veterinarians collect data, process it rapidly and create valuable graphs and charts to allow diagnostic analytics of the pregnancy distribution. This app is used by veterinarians to enhance monitoring and evaluating cowherd breeding season success. Ability to visualize the percentage of cows becoming pregnant each 21 days of the breeding season can provide important information to identify contributing causes for situations when a lower than desired percentage of herd becomes pregnant, or to identify areas for improved reproductive efficiency. Until now, collecting and evaluating information chute-side was difficult.

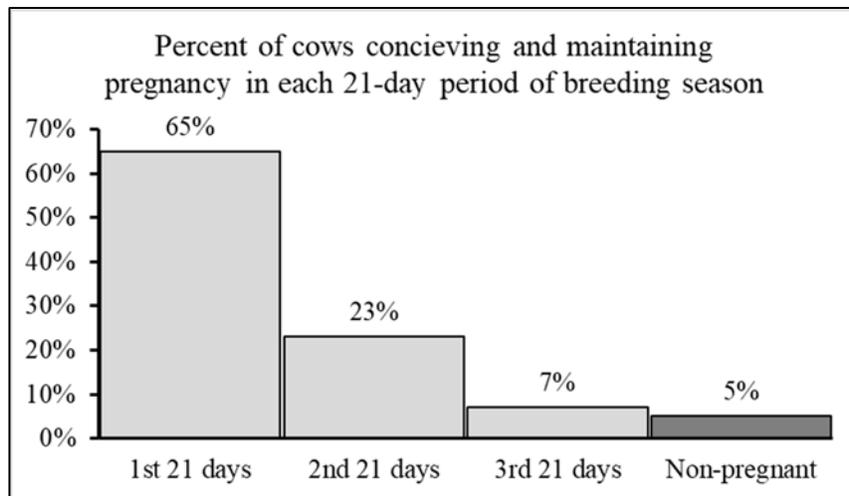


Figure 1. Histogram of the percentage of cows that conceive and maintain a viable pregnancy if nearly all cows ovulate a fertile oocyte during the first 21st days of the breeding season and are bred by fertile bulls and 60 - 70% of available (nonpregnant) cows conceive and maintain a viable pregnancy in each subsequent 21 day breeding period.

Data required by this app are starting and ending dates for the breeding season and an estimate of fetal age for each pregnancy. Additional information such as cow id, cow age, body condition score, and breed (or other descriptor) can be added to enhance value of pregnancy status information. After data are entered, estimated conception dates are generated and histograms created. These pregnancy patterns can help identify most likely contributing factors when investigating herds with a lower than desired percent pregnant.^{1,2}

Veterinarians can be fairly precise estimating fetal age early in pregnancy; however, ability to estimate fetal age accurately decreases as pregnancy progresses.³ Therefore, to confidently place cows within fetal age groups, pregnancy diagnosis should occur ≤ 120 days after breeding season initiation. Ability to place animals within fairly tight 21 day periods is a great advantage for veterinarians when evaluating the herds' recent past and optimizing future herd management.

The ideal distribution for a 63 day breeding season should resemble Figure 1. Producers should strive for nutritional and management systems that allow $\geq 60\%$ of exposed animals to become pregnant in the first 21 breeding season days,⁴ with the majority of remaining animals becoming pregnant in the second 21 day period and no more than 5% of the herd being classified as nonpregnant.

Another way to evaluate pregnancy distribution data is to determine percent of the available (nonpregnant) cattle that become pregnant each 21 day period. As the breeding season advances and pregnant cattle are no longer available to be bred, the herd percentage that becomes pregnant each 21 days is not same as the percentage of available (nonpregnant) cattle that becomes pregnant each 21 days. This important measure is displayed as a table by clicking "**% Pregnancy Success**".

Evaluating the percent of the herd that became pregnant each 21 days does not directly provide accurate visualization of how fertility is changing over the breeding season. The pregnancy distribution displayed in Figure 2 provides evidence that 25% of nonpregnant cows became pregnant in the first 21 day period. However, it is not as clear that in the second 21 day period, 40% of nonpregnant cows became pregnant (30% of herd), and in the third period, 65% of nonpregnant cows became pregnant (30% of herd), and finally, that in the fourth period, 65% of nonpregnant cows became pregnant.

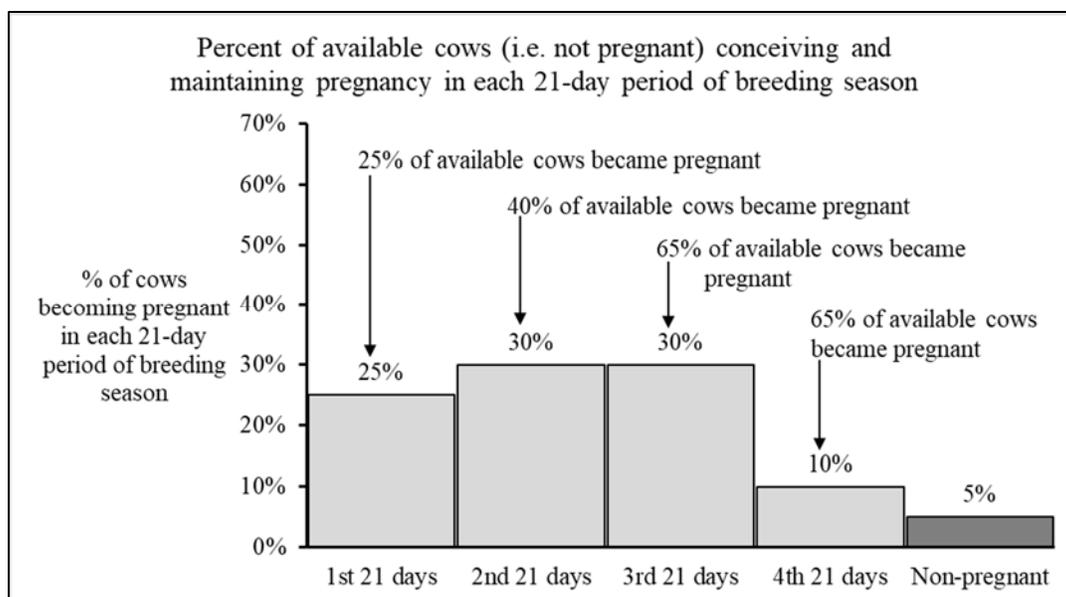


Figure 2. Reporting % Pregnancy Success as a percent of available (nonpregnant) cows at the start of each 21 day period that became pregnant within each 21 day period provides a more direct indication of changing fertility over the breeding season.

Based on expected pregnancy success when cow and bull fertility is optimal, the “% Pregnancy Success” goal should be between 60 and 70% for every 21 day period of the breeding season. Using the herd represented in Figure 2, by the third 21 days, there is no problem with fertility in cows or bulls. The % Pregnancy Success values clearly indicate that reproductive problems in this herd occurred during the first 2 cycles, whereas fertility was optimal in the last 2 breeding season cycles.

BCI pregnancy analytics app: interpreting charts and tables

At pregnancy diagnosis, veterinarians can estimate fetal age and evaluate palpable or ultrasonographic characteristics of nonpregnant reproductive tracts (Figure 3). If low pregnancy percentage is due to failure to conceive due to cows not resuming fertile cycles postpartum or bulls failing to deliver fertile semen, reproductive tract examination should reveal characteristics of a nonpregnant uterus with no indication of previous pregnancy or uterine pathology. Typical timing of pregnancy diagnosis relative to reasons for early gestation loss due to noninfectious (e.g. heat or transportation stress) or infectious (e.g. Trichomoniasis) causes may or may not be associated with still-detectable uterine involution or pathology. Because infectious agents or toxins causing pregnancy losses often occur in late pregnancy just prior to or following examination for pregnancy status, examination of nonpregnant reproductive tracts due to recent abortion should reveal some reproductive tracts with characteristics of involution or uterine pathology.

Once all data collected at pregnancy diagnosis are organized, in-depth and efficient evaluation of herd reproductive success can be conducted. Reasons for low pregnancy percentage during any 21 day period can be placed into one of 3 categories: 1) inadequate percentage of females having fertile estrous cycles; 2) bulls not able to deliver adequate amounts of fertile semen; or 3) infectious or noninfectious agents prevented or ended pregnancy. Pregnancy analytics app charts and graphs, along with reproductive tract and cow body condition physical examination findings at pregnancy diagnosis, can guide history questions, further physical examination, herd record evaluation, and diagnostic laboratory testing to assist evaluation of possible rule-outs as likely or unlikely causes of undesired pregnancy distributions.

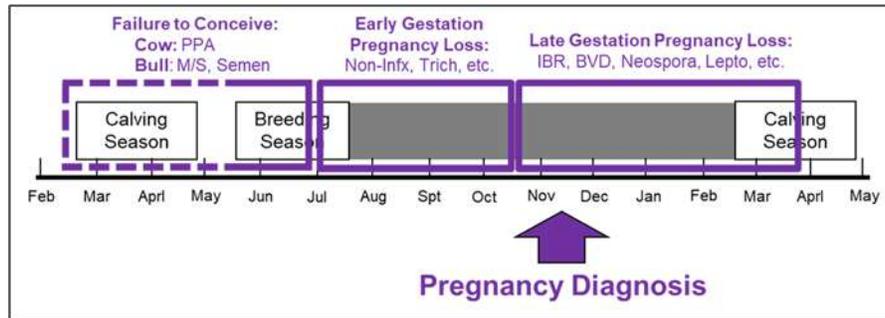


Figure 3. Timing of pregnancy diagnosis relative to reasons for low pregnancy percentage indicates that failure to conceive due to cows not resuming fertile cycles postpartum or bulls failing to deliver fertile semen to cow’s reproductive tract occurred many weeks prior to examination, early gestational loss due to noninfectious (e.g. stress) or infectious causes (e.g. Trichomoniasis) occurred a few weeks to a few months prior to examination, and late pregnancy loss due to infectious agents or toxins occurred just prior to or following examination.

Inadequate percentage of animals were cycling by the 21st day of breeding

Although Figure 1 depicts an ideal herd, many times evaluation of herd pregnancy status data reveals a distinctly different pregnancy distribution. Figure 4 illustrates a very common distribution. In this situation, the percentage of nonpregnant cows would not necessarily indicate a problem if the breeding season lasts long enough; therefore, further evaluation is needed to begin a diagnostic workup.

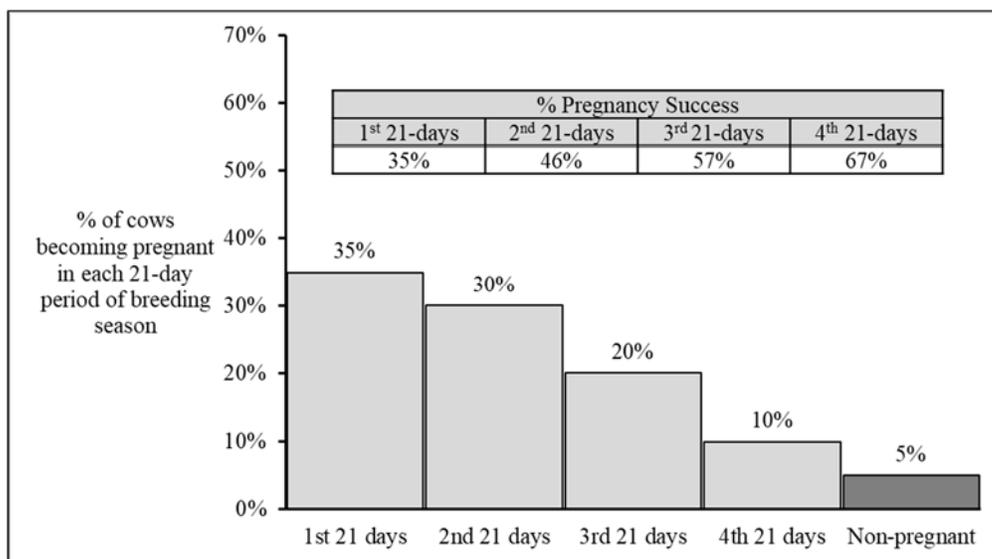


Figure 4. Typical pregnancy distribution for herd with 50% of cows cycling by end of first 21 days of breeding season.

A common reason for a pregnancy distribution similar to Figure 4 is that a similar pregnancy distribution the previous year resulted in many cows calving in third or later 21 day period of the calving season. The typical amount of time from calving to resumption of fertile cycles (postpartum anestrus period) for 90% of a herd’s mature cows is 60 - 80 days⁵ and for primiparous cows, it is closer to 100 - 120 days.⁶ If the breeding season begins on the same date as the previous year (and the breeding season lasts 63 days), the breeding season will commence 62 - 82 days postpartum and end 125 - 145 days postpartum for cows calving in first 21 day period of previous calving season.

Therefore, all early-calving cows (including primiparous cows) are expected to express estrus and be bred several times during the breeding season. Cows calving in the second 21 day period will be

41 - 61 days postpartum at the start of breeding season and 104 - 124 days past calving 63 days later. Once again, this timing should allow mature cows to resume cycles and have multiple opportunities to be bred during the breeding season. Primiparous cows calving in the second 21 day period should also resume cycling early enough in breeding season to have 1 or 2 opportunities to be bred. In contrast, for cows that calve in the fourth 21 day period, calving has just finished as breeding begins and for those in the fifth 21 day period, the breeding season begins prior to the time they calve. Limited interval from calving to breeding season initiation essentially eliminates the potential for nursing cows, and in particular, primiparous cows, to rebreed early in the breeding season.

Without implementing culling, nutrition, and heifer development changes in herds with flat pregnancy distributions similar to the herd depicted by Figure 4, it is very difficult to positively influence the percentage becoming pregnant in first 21 days of subsequent breeding seasons. Reasons that herds with a previously ideal calving distribution can deteriorate to a less-than-ideal situation includes animals too thin at calving, poor postpartum cowherd nutrition, subfertile bulls, or infectious or non-infectious pregnancy loss.^{5,7,8,9}

Bulls failed to deliver adequate amounts of fertile semen

If reproductive performance is initially adequate - indicating that conception occurred and pregnancy was maintained early in the breeding season, veterinarians can assume that fertile bulls were mating fertile, cycling cows, the herd was free of pregnancy wasting disease, and the postpartum anestrus period and energy reserves (as indicated by body condition score) were not problematic. A sharp decline in *Percent Pregnancy Success* during the breeding season should initiate an investigation to identify potential testicular, reproductive tract, or musculoskeletal problems that prevented production or delivery of fertile semen or whether herd replacements brought in after start of breeding season could have introduced venereal disease.

Figure 5 illustrates a problem that is quite common in herds with 1 bull for each breeding pasture. Although multiple-bull breeding pastures are more resilient to breeding failure due to bulls being unable to successfully breed cows compared to single-bull pastures, because of potential problems arising from injuries due to bull-on-bull fighting, social dominance by subfertile bulls, and isolation of groups of cows in extensive breeding pasture without 1 or more bulls present, multiple-bull pastures can also have poor reproductive efficiency due to bull problems and can have a pregnancy distributions similar to Figure 5.

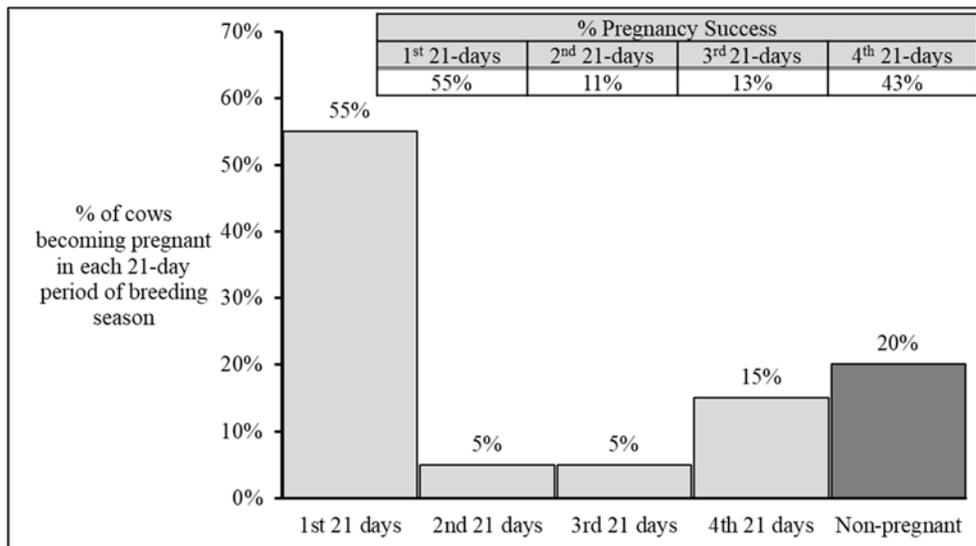


Figure 5. Pregnancy distribution in a herd where a high percentage of cows are cycling at breeding season initiation and bulls are successfully breeding cows, but acute onset of bull infertility occurring late in the first 21 day period or early in the second 21 day period of the breeding season (e.g. injury, disease, etc.) resulted in reduced herd fertility followed by partial recovery.

The breeding season in this example (Figure 5) starts with a high percentage of fertile cows cycling and good bull fertility. Because 55% of the herd becomes pregnant in the first 21 day period, veterinarians can be confident that the prebreeding feeding/supplementation program offered adequate nutrients for a fairly high level of reproductive performance. It is also evident that bulls were able to cover the breeding pasture, find cows displaying estrus, and successfully breed cycling cows.

The dramatic decrease in pregnancy percentage during the second 21 day period is strong evidence for bull infertility due to testicular or musculoskeletal insult.¹⁰ Incremental increase in the percentage of available (nonpregnant) cows bred in each subsequent 21 day period in Figure 5 indicates bull fertility is gradually returning.

In a situation where veterinarians evaluated overall pregnancy percent but not pregnancy distribution for the herd depicted by Figure 5, the percentage of nonpregnant cows would indicate herd fertility problems, although the cause of high nonpregnancy would not be evident. With limited information, nutrition or cow fertility could be suspected. And, as with this example, a bull may pass a breeding soundness examination both before and after being placed in the breeding pasture without revealing that a fertility problem existed during breeding season. By categorizing and displaying information gained at pregnancy diagnosis, the problem cause becomes more obvious.

A bull breeding soundness examination (including a through physical examination) at the time the breeding season problem is discovered, may supply information about penile, testicular, foot and leg or other musculoskeletal problems that commenced during the breeding season. However, lack of identifiable pathology following the breeding season does not rule-out a physical (locomotion, mounting, intromission) or semen quality problem several weeks to months earlier.

Infectious or noninfectious agents prevented or ended pregnancy

In situations when early pregnancy loss leads to negative pregnancy distribution effects, the problem occurred after breeding season initiation and before pregnancy diagnosis. In addition to the pregnancy distribution effect of pregnancy loss, in some situations, nonpregnant cows may exhibit palpable evidence of an involuting uterus at pregnancy diagnosis.

Noninfectious pregnancy loss very early in pregnancy due to environmental stress should not result in uterine pathology and would not be expected to have negative carry-over effects in the next 21 day breeding season period. In contrast, noninfectious pregnancy loss after maternal recognition of pregnancy (~ 13 days after estrus) will delay returns to normal fertility until after the embryo is resorbed or expelled and hypothalamic-pituitary axis has resumed normal estrous cycle activity – which may be later than the 21 day period following initial conception. Early, noninfectious pregnancy loss that occurs before pregnancy is detectable by transrectal palpation or ultrasonography is unlikely to be differentiated from failure to conceive.

Infectious pregnancy loss may result from fertilization failure or very early embryonic death; therefore, transrectal palpation or ultrasonographic examination is indistinguishable from failure to conceive or early noninfectious pregnancy loss. However, because many common causes of infectious pregnancy loss in North America have peak incidence after mid-gestation when pregnancy is typically diagnosed, it is expected that palpable evidence of previous pregnancy will be detected. The length of time that palpable evidence would be evident is influenced by the stage of pregnancy at the time of pregnancy loss and whether or not the loss was accompanied by uterine pathology.

Infection with *Trichomonas foetus* (Trich), a protozoa transmitted during breeding, is an important cause of pregnancy loss in North America because it is diagnosed in many cattle-dense areas and can cause a high percentage of exposed cows to resorb or abort their pregnancy. The pregnancy distribution of a herd infected with Trich will vary depending on what the distribution would have been without infection and the timing of Trich introduction.

If Trich entered herd prior to breeding season initiation so that a high percentage of bulls are already infected, cows will become pregnant at a time similar to last year's breeding season, but infected cows are likely to lose their pregnancies ~ 15 - 80 days into pregnancy. A period of female infertility is expected to last for another 2 - 6 months as a result of infection. The magnitude of loss is expected to

approach 30 - 50% of exposed cows. However, if Trich entered the herd during breeding season or fewer bulls were infected at the start of breeding season but the number of infected bulls increased as the breeding season progressed, then the pregnancy distribution is greatly influenced by what the distribution would have been without Trich exposure, and the speed at which additional bulls became infected.

Other causes of early pregnancy loss (e.g. *Campylobacter fetus* ss *venerialis*, Bluetongue virus, *Leptospira borgpetersenii* serovar *hardjo* type *hardjobovis*, bovine viral diarrhea virus) will have a similar effect on the pregnancy distribution, although magnitude of pregnancy loss is not expected to be as high as with Trich.^{11,12} Infectious and toxic causes of pregnancy loss commonly expressed in mid to late pregnancy include: Bovine Herpes virus 1 (Infectious Bovine Rhinotracheitis), bovine viral diarrhea virus, *Neospora caninum*, *Leptospira* sp., pine-needle toxicosis, and others.^{13,14} Pregnancy losses in mid to late pregnancy are likely to occur after pregnancy diagnosis time and the effect is not limited to a single period of the pregnancy distribution. If pregnancy losses occurred prior to pregnancy diagnosis, evidence of that loss is likely to be apparent during transrectal examination of the nonpregnant uterus of some of the affected cows.

Second-level analysis of pregnancy data

To capture more information from fetal aging, the distribution of breeding dates can be analyzed not only by 21 day intervals, but also by category within 21 day intervals. For example, the herd depicted in Figure 6 has a pregnancy percentage of 94.5%, which meets the goal for a 63 day breeding season. In addition, 61.8% of the herd became pregnant during the first 21 days of the breeding season – which exceeds the 60% cutoff associated with good cow and bull fertility at breeding season initiation. From these observations, one could classify this herd as having normal fertility, with no nutritional or reproductive management problems.

Looking at *Percent Pregnancy Success*, during the first 21 days of the breeding season, 62% of available cows became pregnant. In the second 21 days, 55.6% of nonpregnant cows became pregnant; which is 21% of the herd. In the third 21 days, 68% of available cows became pregnant; which is 11.5% of the herd. These measures of reproductive success are not alarming, but there is an indication that fertility may be suboptimal during the second 21 days and closer examination of data is warranted.

If data collected at pregnancy diagnosis for the herd depicted in Figure 6 are further analyzed by age categories for each 21-day period (Figure 7), the pregnancy distribution for primiparous cows indicates a clear problem. Diagnostic information provided by *Percent Pregnancy Success* indicates that whereas mature cows perform very well throughout the breeding season and primiparous cows performed well during in first 21 days, pregnancy success dramatically decreases during the second 21-day period for primiparous cows before returning to 70% for the final 21 days of breeding.

That primiparous cows performed well the first 21 days of the breeding season is important to recognize because my bias when primiparous cows perform worse than mature cows is that the deficit is because it took them longer to begin fertile cycles after calving and they performed poorly early in the breeding season. However, information provided by the Pregnancy Analytics App for this herd indicates that primiparous cows did not have delayed return to estrus; in fact, the problem was confined to the second 21 day period. Without second-level analysis, I would probably assume that heifers were too thin when they calved or that the producer should move the heifers' breeding season so that they could calve earlier than mature cows. Because of information provided by second-level analysis of data collected at pregnancy status determination, I am able to identify “**which**” cattle were not pregnant and “**when**” during the breeding season fertility was reduced; for this example, I am most interested in investigating bull issues confined to the second 21 day period of the breeding season.

Having this type of analytics available immediately after pulling the palpation sleeve off is not diagnostic *per se*. However, as I am talking to a producer, I can confine my history questions to primiparous cows during the second 21 days of the breeding season (which for this herd would have been the last 2 weeks of June through the first week of July). If I pursue diagnostic testing, I will focus my testing on bulls in the primiparous cow breeding pasture.



Figure 6. Herd with a good pregnancy distribution that has a hidden problem



Figure 7. Second-level analysis of pregnancy data from a herd with suboptimal fertility not apparent when evaluating overall herd pregnancy distribution.

Conclusion

Information gathered at pregnancy diagnosis is very valuable to both veterinarians and beef producers, particularly if fetal age is estimated within 21 day periods. Despite the importance of reproductive performance to cowherd profitability and sustainability, without an efficient and convenient method to collect and analyze pregnancy status data, that value is difficult to capture. Nutrition, genetics, animal husbandry, reproductive soundness and health influence distribution of pregnancy within a herd. By combining transrectal palpation or ultrasonographic imaging of the reproductive tract to determine pregnancy status with analysis and graphing of information, veterinarians can identify **when** during the breeding season **which specific categories** of cows did not become pregnant. The Pregnancy Analytics App allows a veterinarian's knowledge and skill to be augmented by efficient digital data entry and rapid generation of commonly used herd reproductive assessments to enhance communication between veterinarian and producer. More information about the BCI Pregnancy Analytics App is available at <https://ksubci.org/pregnancy-analytics-mobile-app/>

Conflict of interest

There are no conflicts of interest to declare.

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Postpartum anestrus of beef cows: importance and evaluation

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Abstract

Duration of anestrus period and infertility in postpartum suckled beef cows impact reproductive performance and efficiency of cow-calf operations. Percent cows calving in first 21 days of calving season is higher for herds with shorter (average 50 days) rather than longer (average 80 days) length of postpartum anestrus. Because primiparous cows may have a prolonged postpartum anestrus compared to similarly managed multiparous cows, breeding replacement heifers before mature cows, sometimes referred to as providing a heifer lead time, may increase likelihood that primiparous cows have resumed fertile estrous cycles and have opportunity to conceive during first 21 days of breeding season subsequent to first parturition. While having a 42 day heifer lead time resulted in greater pregnancy success and average weaning weight compared to no lead time, ~ two-thirds of improvement in outcomes was realized with a heifer lead time of only 21 days. Determining length of postpartum anestrus following first and subsequent pregnancies can assist veterinarians and producers to develop optimal herd management strategies.

Keywords: Postpartum, anestrus, infertility, bovine

Introduction

Postpartum period of infertility is time from calving until a cow resumes fertile estrous cycles and can become pregnant again. There are 2 important events place during postpartum period; uterus must return to a pre-pregnancy state and the hormones that control estrous cycle must resume normal function to allow fertile ovulations. During postpartum period, cow has highest nutritional demand of the year as lactation is initiated and daily milk production peaks at ~ 60 - 90 days postpartum, uterus undergoes involution, and fertile estrous cycles are resumed, enabling mating and conception for subsequent calving season.

Uterus decreases in weight from 20 - 25 pounds at parturition to < 1 pound by 25 days postpartum. During first few days after parturition, uterus is normally thick-walled and tonic, but if infection is present, it may have a thinner wall and atonic. Uterine involution is not affected by suckling and is similar in beef and dairy cattle. Normal involution process begins shortly after parturition, upper two-thirds of caruncles undergo necrosis due to loss of blood supply and necrotic tissue is sloughed and expelled as part of the uterine lochia.¹ Uterus usually is near nonpregnant size by 3 - 4 weeks postpartum, with previously pregnant horn still slightly larger.² Parturition is followed by an ~ 3 week interval when conception is not possible. Estrus and ovulation seldom occur together during this period and, if fertilization occurred and embryo reached uterus, placentation would be virtually impossible. Period of infertility is followed by 2 - 3 weeks when fertility is possible, but not optimal.² Uterine involution appears not to be a barrier to fertility after 5 - 6 weeks postpartum in cows, unless delayed by inflammation or infection.²

Anestrus is major component of postpartum infertility and is affected by several minor factors: season, breed, parity, dystocia, presence of a bull, and carryover effects from previous pregnancy and 2 major factors (suckling and nutrition).³⁻⁶ As reported earlier,⁵ for spring-calving herds, duration of postpartum anestrus decreased with increasing Julian day.⁷ Season may truly have a role in modifying duration of postpartum anestrus through light stimulation of pineal gland.⁸ Presumably, differences in duration of postpartum anestrus associated with season or Julian day could also be related to quality and quantity of available forage.

Suckling has a dramatic effect on postpartum interval. In suckled cows, first ovulation is delayed to 30 days or more postpartum. Cows that have their calves weaned at birth have shorter postpartum interval than do cows that are suckled. If calves are weaned at some time after birth but before estrous cycles begin, cows will return to estrus in a few days. Postpartum intervals can be decreased by complete

weaning and to a less predictable extent, by short-term weaning (48 hours) or restricted suckling.⁹⁻¹² Conception success is lower up to 40 days after parturition, as function of first corpus luteum as measured by serum progesterone concentrations is less than subsequent cycles, and with reduced estrus signs.³ Normal corpus luteum function during an early postpartum estrous cycle can be obtained by pretreatment with a progestin.¹³

A cow's nutritional demand is highest of production cycle during postpartum period primarily due to demands of lactation, which peaks at ~ 60 - 90 days after calving.^{14,15} Quantity and quality of feed intake, nutrient reserves stored in the body and competition for nutrients from other body functions besides reproduction (lactation, growth, etc.) are all factors that affect length of time required to resume cycling. Body weight and condition score, are good indicators of energy status and rebreeding performance after calving. Inadequate precalving and/or postcalving energy or protein nutrition extends length of postpartum anestrus.

Postpartum anestrus lasts an average of 55 - 65 days for multiparous cows in good body condition (longer if low body condition).^{7,16-19} Regardless, recognize that full consideration of average postpartum length means that ~ 50% of cows in good body condition have not resumed fertile cycles by 55 - 65 days. Postpartum anestrus lasts an average of 80 - 100 days for primiparous cows in good body condition.^{20,21} Dystocia will increase length of postpartum anestrus. Adverse effects of dystocia can be overcome at least partially by providing early obstetrical assistance.

Importance of length of anestrus

There are several reproductive constraints in beef cattle production. First is that when a fertile cow and a fertile bull mate, about 60 - 70% of time a viable calf will be detected at determination of pregnancy status during midpregnancy.²² However, ~ 30% of time following a fertile mating, early embryo dies within first 13 days. When an embryo is lost before maternal recognition of pregnancy (~ 13 days after ovulation), cow will display estrus and ovulate an oocyte about 21 days after her last ovulation and have another 60 - 70% probability of achieving and maintaining pregnancy.²² Another constraint is that there are 283 days of pregnancy and 365 days in a year, which means that a cow must achieve a viable pregnancy within 82 days after calving in order to maintain a 365 day calving interval.

Based on these 2 constraints, best herd reproductive performance that can be expected would be depicted with a histogram where 65% of the herd becomes pregnant in first 21 days. This would indicate that nearly every cow has resumed fertile estrous cycles by the 21st day of breeding season and bulls are able to successfully mate cows. Cows that are fertile during first 21 days of breeding season but fail to maintain a pregnancy from first mating, are expected to express estrus and ovulate approximately 21 days after their first ovulation and will have another 60 - 70% probability of establishing a viable pregnancy which results in another 23% of herd becoming pregnant in the second 21 days of the breeding season. Finally, if cows fail to establish viable pregnancies after 2 fertile matings, ~ 60 - 70% of remaining cows become pregnant in the third 21 days of breeding season, leaving the herd with 5% nonpregnant cows after a 63 day breeding season (Figure).

In order to investigate effect of length of postpartum infertility on percentage of cows in a herd that conceive and maintain a viable pregnancy during first 21 days of breeding season, average age at weaning and total weight of calves weaned over a 10 year interval, a deterministic, dynamic systems model was developed²³ to compare 22 combinations of multiparous and primiparous postpartum anestrus length. Model compared herds that averaged 50, 60, 70, and 80 day postpartum anestrus for multiparous cows and 50, 60, 70, 80, 90, 100, and 110 day postpartum anestrus for primiparous cows. Percent cows calving in first 21 days of calving season is higher for herds with shorter (average 50 days) rather than longer (average 80 days) length of postpartum anestrus.²³ Earlier calving cows produce heavier calves at weaning, as demonstrated by a model that calculated weaning weights that averaged 65 lbs heavier for herds with 50 day average duration of postpartum anestrus compared to herds with 80 day average duration of postpartum anestrus.²³ In addition, cows with a 70 day or greater length postpartum anestrus were less likely to become pregnant in a 63 day breeding season.²³

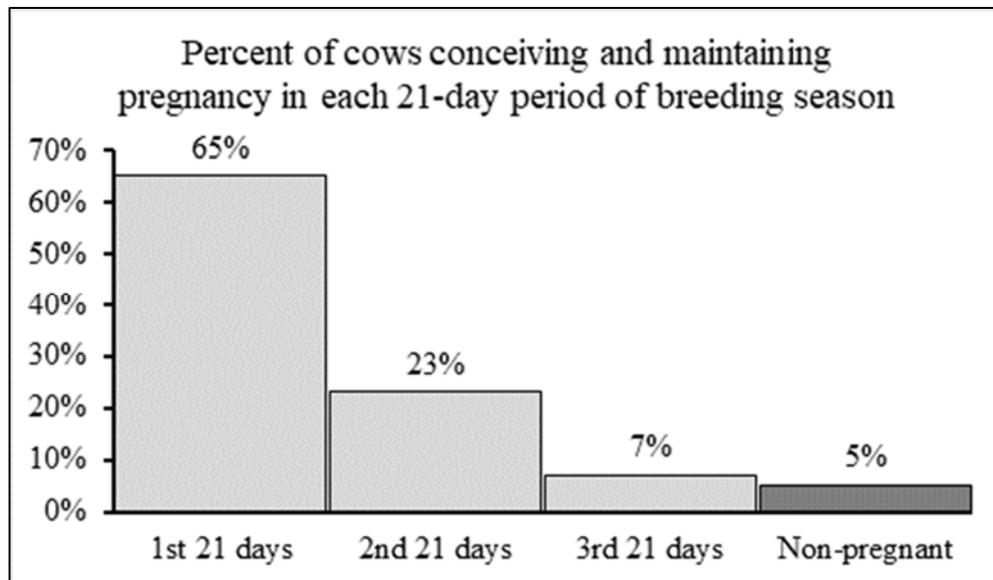


Figure. Histogram of the percentage of cows that conceive and maintain a viable pregnancy if nearly all cows in the herd ovulate a fertile oocyte during the first 21st days of breeding season and are mated by fertile bulls and 60 - 70% of available (nonpregnant) cows conceive and maintain a viable pregnancy in each subsequent 21 day breeding period.

Because postpartum anestrus lasts longer in primiparous cows than similarly managed multiparous cows, nulliparous heifers must be bred to calve before multiparous cows to enable them to ovulate a fertile oocyte during first 21 days of their second breeding season. Some cattle production consultants recommend that cow-calf producers breed nulliparous cows before primiparous and multiparous cows, sometimes referred to as providing a heifer lead time. A deterministic, dynamic systems model was used to evaluate herds with an average 60 day postpartum anestrus for multiparous cows and one of 42 combinations of 60, 70, 80, 90, 100, or 110 day average postpartum anestrus for primiparous cows and 0, 7, 14, 21, 28, 35, or 42 day heifer lead times.²⁴ Whereas having a 42 day heifer lead time resulted in greater pregnancy success and average weaning weight compared to shorter lead times, approximately two-thirds of improvement in outcomes was realized with a heifer lead time of only 21 days.²⁴ In addition, primiparous cow cohorts with average length of postpartum anestrus of 70 days or longer should be bred to calve ahead of cows or they will contribute to negative annual reproductive momentum and an unacceptable percentage of primiparous cows will fail to become pregnant in a 63 day breeding season.²⁴

Evaluation of length of anestrus

Duration of postpartum anestrus can be a major contributing factor to herds with poor reproductive performance and low calf weight weaned per cow exposed over time and should be a rule-out when investigating lower than desired reproductive efficiency. It appears that maintaining an average duration of postpartum anestrus for multiparous cows to < 60 days results in improved production outcomes when compared to a longer postpartum anestrus. In addition, herds with a shorter duration of postpartum anestrus are more resilient to negative effects of various unpredictable and sporadically occurring adverse production events.

Quantifying a herd's average length and range of postpartum anestrus could be useful to veterinarians seeking to improve reproductive management and performance of beef cow-calf herds. An estimate of average and range of anestrus duration could be determined by selecting a sample of cows in a herd that have calved at various time points in calving season and monitoring them for estrus signs and behavior to establish resumption of estrous cycles. Determining duration of postpartum anestrus for age-specific cohorts such as primiparous versus multiparous cows may have additional value. Several

technological aids (heat-detection patches, chin-ball markers, and electronic, indirect monitoring technologies) were developed to assist with monitoring cattle for onset of estrus. Many of these technologies are inexpensive and are easily applied to cattle.

Conflict of interest

There are no conflicts of interest to declare.

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Bull reproductive development and sperm production enhancement

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Abstract

The onset of puberty and daily sperm production of bulls are fundamental aspects that impact natural breeding and semen production centers. Critical molecular and endocrine aspects of testicular development, spermatogenesis and functions are reviewed. Methods to enhance sperm production (gonadotropin supplementation, immunization against inhibin and induction of temporary hypothyroidism) and findings are discussed.

Keywords: Spermatogenesis, Sertoli cell, endocrinology, puberty, hypothyroidism

Introduction

Important aspects of bull testicular development and sperm production have been reviewed.¹⁻³ Breeding soundness examinations of thousands of bulls are performed every year before they are used for natural breeding or enter artificial insemination centers. However, on their first examination, approximately 20 - 25% of yearling beef bulls fail to meet minimum standards for testicular size and sperm morphology.^{4,5} For high genetic value bulls entering artificial insemination centers for semen collection and cryopreservation, maximum sperm production is required at each collection.

Development of reproductive system and in particular testicular function, starts during fetal life and continues through postnatal period until puberty (Table). These developmental periods are primarily affected by genetics and environmental factors. Testicular function and spermatogenic efficiency are mainly influenced by factors during prenatal period, prepubertal growth phase and post-pubertal maturation phase. Understanding bull gonadal development facilitates optimal management of seedstock dams and young bulls. Objectives are to review bull developmental biology, factors affecting spermatogenesis and potential ways to improve sperm production.

Development of reproductive system in the fetus

Understanding testicular differentiation and development has facilitated substantial improvement in recent years using molecular tools and advancements in genomics. Differences in gene expression can be detected as early as blastocyst stage.^{6,7} Differentiation of inner cell mass into somatic germ layers (ectoderm, endoderm, and mesoderm) follows hatching. Hypothalamus, pituitary gland, and penis derive from ectoderm, whereas gonads, epididymides, ductus deferens and urinary system derive from mesoderm. Somatic germ cells are pluripotent except some yolk sac inner lining cells that differentiate into primordial germ cells (PGCs). Undifferentiated gonad forms after passive or amoeboid migration of PGCs into genital/gonadal ridge. This phenomenon is likely controlled by several molecular signals that are not yet completely understood. High mitotic index is recognized in PGCs and they rapidly populate genital ridge; 1000 - 2000 PGCs are already present in genital ridge by 25 days and gonads can be identified at 28 - 29 days of pregnancy. Several key player genes (Wilms tumor-1 factor, *Lim1* transcription factor, and Steroid factor) are identified in gonadal development.^{8,9} Wilms tumor-1 factor and zinc finger like transcription factor (produced by Sertoli cells) have important roles in testicular cord assembly and regulating development of fetal Leydig cells and peritubular myoid cells.¹⁰

Differentiation of fetal testes is determined by SRY gene (Y chromosome sex determining region). Expression of SRY begins on day 37 and peaks on day 39.¹¹ This gene encodes for a protein of high mobility group and regulates several other genes involved in gonadal differentiation into testis. One of these genes, *SOX9*, is essential in differentiation of fetal Sertoli cells. Furthermore, *GATA4* (required for expression of SRY gene and formation of genital ridge) and *DMRT* are also implicated.

Table. Chronology of major events during fetal and postnatal development of bull reproductive system

Period	Major events
Embryonic and fetal development	
Blastocyst	Differences between male and female embryos in gene expression
Hatching	Primordial germ cells derived from inner lining of yolk sac
Day 25	1000 - 2000 primordial germ cells migrate to genital ridge
Days 28 - 29	Presence of undifferentiated gonad
Days 37 - 39	SRY expression
Days 41 - 42	Differentiation of male gonad, testicular chords, fetal Leydig cells and Sertoli cells
Day 47	Masculinization of external genitalia due to effects of testosterone and androstenedione from fetal Leydig cells
Day 50	Start of paramesonephric ducts regression
Days 56 - 58	Appearance of seminal vesicles and prostate
Day 60	Differentiation of scrotum
Day 70	Branching of seminal vesicles, differentiation of epididymis, stabilization of mesonephric ducts
Day 80	Complete regression of paramesonephric duct; testes begin transabdominal phase of descent
Day 90	Presence of major components of male reproductive system
Day 110	Formation of epididymis, testis begin inguinoscrotal phase of descent
Day 120	Testicular descent into scrotum
Postnatal development	
Month 1	Degeneration of fetal Leydig cells, increased adult Leydig cells and undifferentiated Sertoli cells; proliferation of prespermatogonia
Month 5	Appearance of primary spermatocytes; seminiferous tubule lumens begin to form
Months 5 - 8	Presence of primary and secondary spermatocytes in seminiferous tubules
Months 7 - 9	Complete detachment of penis from prepuce
Months 8 - 10	Presence of mature sperm in seminiferous tubule lumens
Months 8 - 12	Puberty: ejaculate with ≥ 50 million sperm and $\geq 10\%$ progressive motility
Months 12 - 16	Maturation

Differentiation of bovine testis starts at 41 - 42 days. At this stage, testicular cords lack a lumen and consist of undifferentiated Sertoli cells. By 60 - 70 days, rete testis appears at end of testicular cords and become connected to each other. Concurrently, first generation of fetal Leydig cells (from mesonephros) appear in mesenchyme. After birth, fetal Leydig cells degenerate and are replaced by adult Leydig cells.

External genitalia become masculinized by day 47 under effects of testosterone and androstenedione produced by Leydig cells. By 60 days, scrotum is well developed. Paramesonephric ducts start regressing by day 50 and are completely regressed by day 80. Seminal vesicles and prostate appear by 56 - 58 days.¹² By end of first trimester, all components of reproductive system are present except for epididymis (begins to form at 110 days.¹³) Internal and external genitalia complete development occurs after 100 days. Descent of testes within scrotum occurs at 100 - 120 days of pregnancy. Testicular descent is mediated by insulin-like peptide 3 (from fetal Leydig cells) that initiates transabdominal phase around 80 - 90 days of pregnancy. Inguinoscrotal phase of testicular descent is mediated by androgens. This early maturation of hypothalamo-pituitary gonadal axis in bovine has been confirmed. Concentrations of insulin-like peptide 3 and testosterone in maternal plasma are significantly higher at 4 and 8 months of pregnancy in cows carrying a male versus female fetus.¹⁴

Prepubertal development

Onset of puberty in bulls is highly dependent on nutrition and body growth. Slow growth is correlated to slower testicular development and delayed puberty. In some breeds, scrotal circumference is highly correlated to body weight.¹⁵ At birth, testes are composed of solid (no lumen) chords comprised of primordial germ cells, fetal Leydig cells and undifferentiated Sertoli cells (Figure 1).¹⁶ Accessory sex glands are not functional and penis is adherent to prepuce. Fetal Leydig cells degenerate

in first postnatal month and are replaced by adult Leydig cells. GnRH receptors in anterior pituitary increase significantly from 6 - 10 weeks.¹⁷ Increased frequency of GnRH pulses and corresponding increases in LH concentrations occur during cell differentiation. Number of undifferentiated Sertoli cells increases rapidly.^{16,18} During prepubertal period, serum FSH and inhibin concentrations are high, but decline with onset of rapid testicular growth.¹⁹ FSH has an important role in prepubertal proliferation of Sertoli cells,²⁰ as it increases at 4 - 25 weeks.²¹

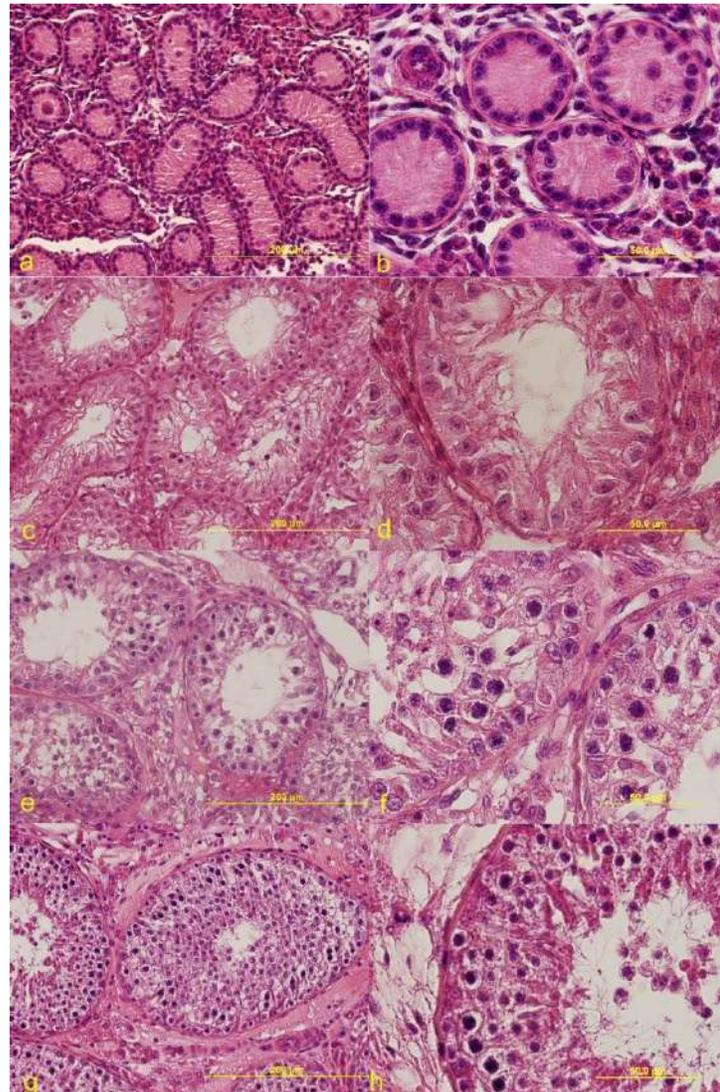


Figure 1: Histological appearance of bull testis at 1 month (a, b), 5 months (c, d), 8 months (e, f), 10 months (g, h); note: development of a lumen at 5 months, initiation of spermatogenesis at 8 months, appearance of sperm in lumen by 10 months. (Left column x 400, Right column x 1000)

Proliferation of prospermatogonia began at 1 month, and primary spermatocytes appeared ~ 5 months.²² Age of puberty was negatively correlated with magnitude of LH secretion in first 5 months of life,²³ a highly heritable trait.²⁴

Detachment of the peno-preputial adhesions occurs ~ 6 weeks prior to puberty. As puberty approaches, testicular and epididymal growth accelerate and become almost linear. Low-frequency LH pulses increase serum testosterone concentrations produced by a rapidly increasing number of adult Leydig cells, promoting establishment of spermatogenesis.¹⁶ Primary and subsequently secondary

spermatocytes are detected 5 - 8 months (Figure 1). Mature sperm are present in seminiferous tubules from 8 - 10 months. Puberty (ejaculate with at least 50 million sperm and at least 10% progressive motility) occurs when scrotal circumference reaches 28 - 30 cm in *Bos taurus*² and 27 - 28 cm in *Bos indicus*.²⁵

Following puberty, bulls enter a phase of maturation characterized by increases in accessory sex gland weights, sperm production and proportion of morphologically normal sperm. This phase of maturation varies considerably amongst bulls, lasting 3 - 4 months in *Bos taurus*.²⁶

Effect of nutrition on early development

Nutrition of the dam profoundly affects testicular development.²⁷ Heifers fed 2.4 x recommended energy and protein requirement in the first 6 months of pregnancy produced calves that had smaller testes and lower serum testosterone concentrations compared to calves from heifers fed 1.9 x or 0.7 x recommended ration.²⁷ Santa Gertrudis bull calves from dams fed low-protein diet had delays in onset of puberty and sexual maturity than those from dams fed high-protein diet.²⁸ However mechanism of this action remains unclear.

Bull calves fed diets to achieve 1.4 - 1.5 kg/day average daily gain reach puberty earlier with greater paired testes weight and daily sperm output.^{2,29,30} In general, with an average daily gain of 1 kg/day, puberty occurs at 8 - 12 months.³¹ Prewaning early development of bulls was largely dependent on maternal milk production. Bull calves born to heifers and aged dams (> 9 years) had smaller scrotal circumference due to lower hypothalamo-pituitary activity.² However, effects of diet on onset of puberty are confounded by genetics and greater feed intake does not consistently result in earlier puberty.³²

It is important to note that a high-energy diet, such as often fed in bull testing stations, may have adverse effects on testicular development and spermatogenesis. Young bulls fed high energy diets have better average daily gain and thicker backfat, but their scrotal circumference may not be altered.^{2,3,34} However, these bulls have more morphologically abnormal sperm due to poor testicular thermoregulation attributed to increased scrotal fat.^{2,33,34}

Enhancement of sperm production

Enhancement of sperm production in valuable bulls is important to satisfy the demand for more doses of semen (particularly sexed semen) for artificial insemination. In addition to care and nutrition of the pregnant dam, several other postnatal strategies were investigated to increase bull sperm production. Unilateral castration increased the contralateral testicular size but failed to increase sperm production. In recent years, our understanding of the role of Sertoli cell as support cell for spermatogenesis, provides new approaches to enhance sperm production.

The role of Sertoli cells in spermatogenesis was studied in a variety of species.^{20,35} Strong positive correlation between Sertoli cell number and sperm production capacity in bulls was established.²⁰ Sertoli cell number correlated with number of spermatogonial stem cell niches in mice, critical for spermatogenic activity and sperm production.^{36,37}

Postnatal expansion of Sertoli cell population was described in several mammalian species as a 2 wave linear increase in Sertoli cell population.³⁷ Termination of Sertoli cell proliferation appears to be an extremely important biological point for manipulating spermatogenesis.³⁷ Increases in testicular estrogen concentrations arrest Sertoli cell expansion in swine but apparently not in bulls.³⁵ In the latter, based on early cytological studies, Sertoli cell number plateaus at 6 - 7 months.²² However, based on cell proliferation marker KI67 and expression of Sertoli cell marker SOX9, Sertoli cell proliferation ceases at 4.5 - 5 months (Figure 2).³⁸

During prepubertal development, Sertoli cell proliferation is stimulated by FSH but inhibited by thyroid hormone concentrations. This relationship between Sertoli cell proliferation and thyroid hormone concentrations was demonstrated in boars³⁹ and rams.⁴⁰ In bulls, thyroxine concentrations were negatively correlated with testicular size at puberty.⁴¹ These observations led to development of hormonal methods during early development to enhance sperm production. These hormonal methods

include immunization against inhibin, GnRH injections, FSH injections, and establishment of a transient hypothyroidism.

Immunization against inhibin in early postnatal period increases serum FSH concentrations, testicular size, and daily sperm production in bulls.⁴² However, it was transient and required multiple vaccinations. There was also a great variability of the response among individuals. Administration of GnRH to prepubertal bulls (120 µg/kg) BID from 4 - 8 weeks hastened puberty by 6 weeks compared to untreated bulls.⁴³

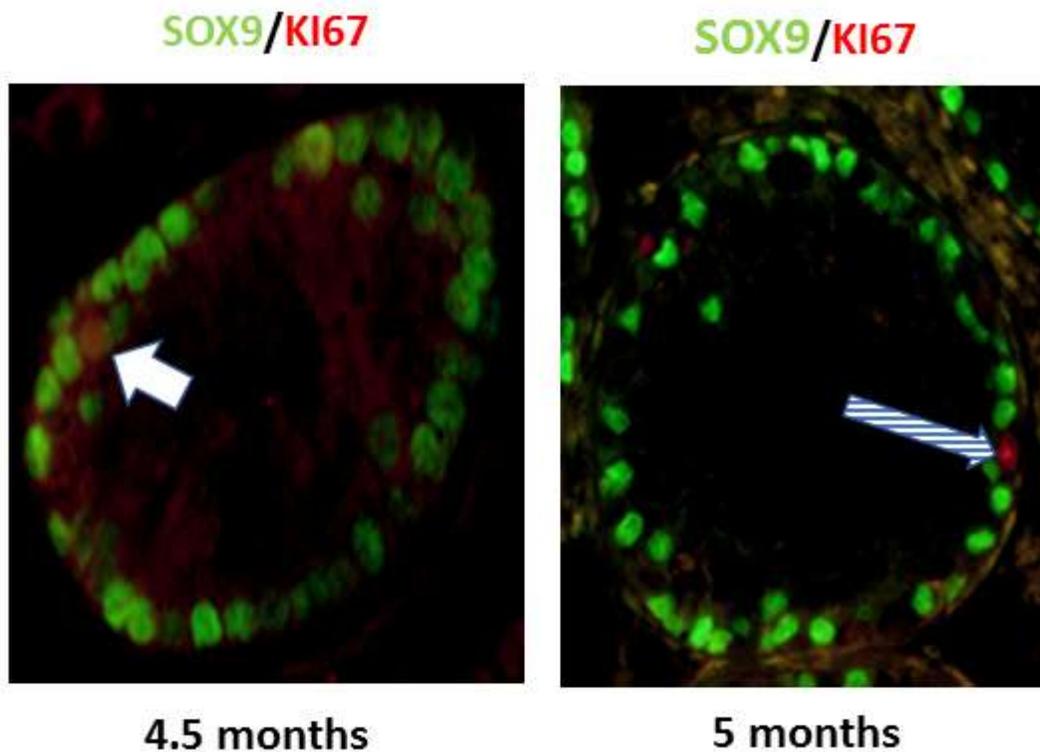


Figure 2. Cross-sectional view of seminiferous epithelium.³⁸ Green cells are SOX9 positive Sertoli cells, red cells are ki67 positive dividing cells. White arrow points to a dividing Sertoli cell stained with both SOX9 and KI67 at 4.5 months. Pattern filled arrow indicates a KI67 positive dividing germ cell at 5 months; note: at 5 months none of the sox9 positive Sertoli cells express KI67.

Bull calves given exogenous FSH every 2 days for 4 - 8 weeks had more Sertoli cells number at 56 weeks and attained puberty 5 weeks earlier than nontreated calves.⁴⁴ In a more recent study, administration of porcine FSH (30 mg, Folltropin-V in 2% hyaluronic acid) from 35 - 91 days increased endogenous FSH at 70 days and Sertoli cell numbers per seminiferous tubule at 93 days,⁴⁵ with increase in endogenous FSH due to a positive feedback loop through Activin A produced by Sertoli cells. FSH-treated bulls had on an average 4 more Sertoli cells per seminiferous tubule section than untreated bulls.⁴⁶ However, there was no difference in spermatogonial cell number.⁴⁶

Potential for enhancement of sperm production after induction of transient hypothyroidism in bulls (based on negative relationship between Sertoli cell proliferation and circulating thyroid hormones concentrations) was investigated.³⁸ To induce hypothyroidism, bull calves received Methimazole (2

mg/kg BW, BID) from 4 - 6 months. Age of puberty was delayed with increased sperm production. Bulls subjected to transient hypothyroidism had 30 - 180% more sperm per ejaculate than control bulls, with no effect on sperm motility, morphology, post-thaw survivability or rates of IVF cleavage and blastocyst development. Treated bulls had a 22% higher mean paired testes weight and 50% higher epididymal weight compared to untreated bulls. Finally, treated bulls had a 2.3-fold increase in number of Sertoli cells per cross section of seminiferous tubule than control bulls. These promising results are impetus for further studies.

Conclusion

In the current era of genomic testing, sire selection can be performed very early in life and bull development and sperm production are increasingly important for efficient use of elite sires. In this context, studies are needed to dissect Sertoli cell-specific role in sperm production and methods to enhance it. Current knowledge on prenatal and postnatal bull development suggests that these phases can be modulated through judicious nutritional supplementation of dams and postnatal hormonal treatments of bull.

Conflict of interest

There are no conflicts of interest to declare.

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Fetal programming: maternal-fetal interactions and postnatal performance

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Abstract

Placental and fetal development is sensitive to direct and indirect effects of maternal environment. Environmental stimulus or insult during critical periods of development (e.g. fetal period) can program lifelong production characteristics of animal, irrespective of their genotype. Several environmental factors negatively impact placental development and blood flow during pregnancy and hinder offspring vigor. Timing of environmental insult or therapeutic intervention during pregnancy is critical, as developmental sensitivity of fetoplacental unit changes from early to late pregnancy. Association between poor growth performance of offspring and decreased blood flow to uterus and placenta during pregnancy was established. Nutrient restriction during early to midpregnancy decreased uterine artery blood flow, leading to compensatory growth of placental blood vessels during exponential fetal growth. Although many studies investigated offspring phenotypes following maternal nutrient restriction or overfeeding during pregnancy, fewer concentrated on enhancing postnatal performance in livestock based on offspring body weight. Survival rate of offspring born at average weight was higher than those with below-average birth weight. In this regard, dietary supplements promoting uterine blood flow increased postnatal growth and weaning weights. Elucidating consequences of specific supplements on continual plasticity of placental functional capacity will allow us to determine important mediators of offspring growth and development.

Keywords: Cattle, development, fetus, nutrition, placenta, sheep

Introduction

Irrespective of animal's genotype, environmental stimulus or insult during a critical period of development can impact phenotype. Exposure to environmental stimulus or insult may establish a permanent postnatal phenotype, resulting in adverse consequences for milk production, carcass yield, feed efficiency, and/or reproductive function.^{1,2} Process of permanently altering animals' phenotype through environmental stimuli is referred as 'developmental programming hypothesis'.² For example, 2 animals with similar genotype but raised in different environments are expected to have differing lifelong phenotypic characteristics, which is further explained by developmental plasticity. Importantly, magnitude of phenotypic change is vastly different between these 2 animals with similar genotype, if their exposure to different environments occurred while they were embryos, fetuses, calves, weaned heifers, or mature cows. Changes in animals' developmental trajectory with lasting consequences were greatest in embryos and fetuses, with decreasing developmental plasticity with increasing animal age. Study of developmental programming during fetal period, an age of high developmental plasticity, is known as 'fetal programming'.

Support for fetal programming concept (strong association between birth weight and lifelong developmental consequences) is growing.^{3,4} For example, low birth weight offspring are at increased risk of morbidity and mortality, slowed postnatal growth, poor body composition (increased fat and reduced muscle growth), metabolic disorders, cardiovascular pathologies, and dysfunction of several organs (ovaries, testes, mammary gland and gastrointestinal tract).^{4,5} Livestock are specifically at risk due to poor nutritional environments during pregnancy (e.g. breeding young, growing, peripubertal dams that are competing for nutrients with fetus). In addition, poor pasture conditions or environmental heat stress in relation to seasonal breeding can decrease nutrient availability for both dam and fetus during critical periods of development.⁶ Although these initial fetal programming studies focused exclusively on offspring (fetal or birth) weight, we now understand that multiple measurements of offspring size at birth can predict developmental trajectory. Therefore, phenotypic changes in livestock production as a result of

fetal programming may be independent of birth weight⁷⁻⁹ with environmental insults during early pregnancy altering phenotypic changes (production) despite no change in birth body weight.¹⁰

Insufficiencies during pregnancy, resulting in reduced fetal growth and development, are detrimental to livestock, where newborns represent next generation of meat and milk producing animals. Several animal models of fetal and placental growth restriction (e.g. maternal nutritional plane, maternal age, heat stress, hypoxic stress, and fetal number) were developed to better unravel relationships among uterine blood flow and offspring development.¹¹⁻¹⁴ Establishment of functional fetal and placental circulation is one of the earliest events during conceptus development^{15,16} and exponential increase in placental exchange is vital for maintaining remarkable growth and development of fetus during last half of pregnancy.¹⁷ Therefore, understanding impacts of maternal environment on placental function is especially relevant to these proceedings, as majority of mammalian livestock raised for red meat production spend 30 - 40% of their life being nourished by placenta. Percent time in each phase of beef production from conception to harvest is depicted in Figure 1. In addition, amount of developmental plasticity of offspring varies during their lifespan, with maximal influences of developmental programming occurring during embryonic and fetal stages (Figure 1).

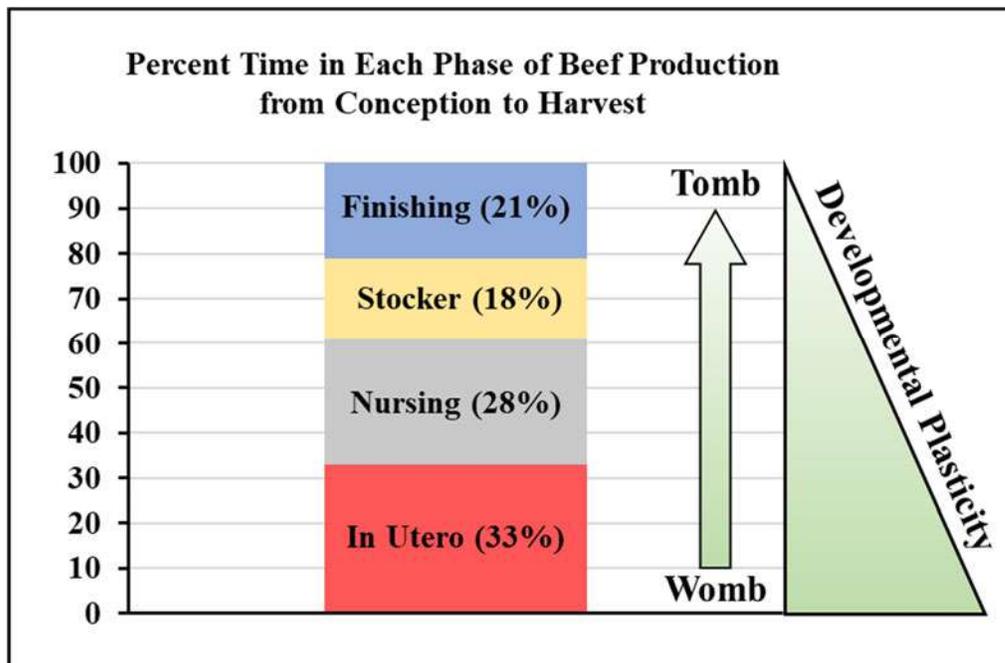


Figure 1. Percent time in each phase of beef production from conception to harvest (womb to tomb), note: nearly one-third of life is spent developing in utero when fetus is most vulnerable to environment, due to increased developmental plasticity.

Fetal growth and organ development

Embryonic period in cattle is defined as time from conception (single cell embryo, as zygote) to completion of organogenesis. This embryonic period typically extends from days 1 - 42 of pregnancy, with highest percent pregnancy wastage occurring during this interval. Fetal period is defined as the remainder of pregnancy from days 42 - 280 at organ differentiation completion (Figure 2).¹⁸ Characterization of bovine fetal growth throughout pregnancy allowed researchers to hypothesize phenotypic changes to offspring that experience specific periods of environmental insults that may perturb normal development in utero. For example, nutrient deprivation or heat stress during days 60 - 120 of pregnancy will undoubtedly have different impacts on fetal development compared to similar environmental insults during days 180 - 240 of pregnancy. This is where timing becomes a critical component of fetal programming outcomes. In addition to extrinsic environmental effects altering fetal growth, several inherent intrinsic mechanisms are associated with fetal growth and development. During

days 70 - 100 of pregnancy, fetal weight across several breeds of cattle increased ~ 10 grams per day.¹⁹ Further along in pregnancy (days 200 - 250), rate of fetal growth increased to ~ 200 - 300 grams per day; however, absolute growth of late term fetus declined to 100 grams per day, which may be due to inherent function of fetus exceeding capabilities of uteroplacental exchange near term. Alternatively, fetus may be secreting or altering its own hormone profiles near term, favoring proper maturation and differentiation of organs overgrowth.

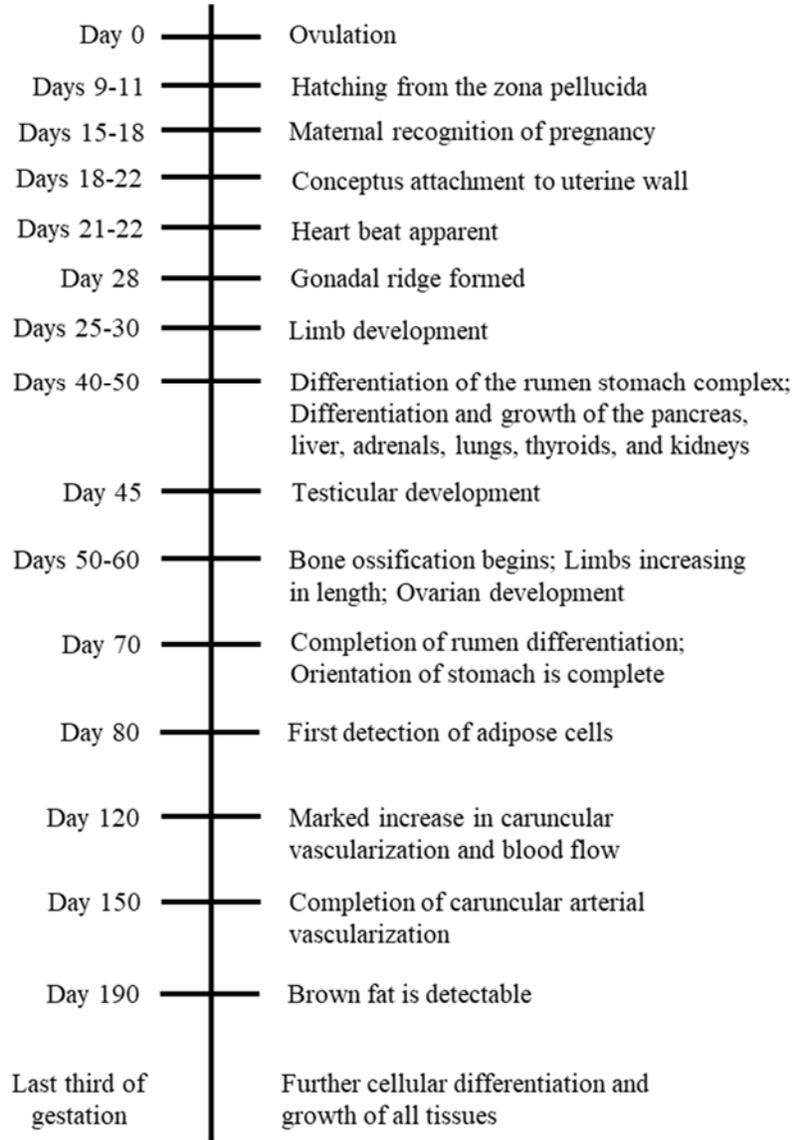


Figure 2. Timeline of bovine fetal development.¹⁸

Bovine fetal growth typically lags allantoic fluid volume and expansion of chorioallantoic membrane.^{19,20} Nutrient partitioning by uteroplacenta and continuous supply of nutrients is a prerequisite for fetal growth. Chorioallantois expansion over endometrium allows for maximal surface area for nutrient exchange during development, which should support acceleration in fetal growth. There was significant positive correlation for total amniotic fluid volume and fetal weight, but no correlation for total allantois fluid volume and fetal weight.¹⁹ Cloning of cattle by somatic cell nuclear transfer allowed researchers to further define this association between fetal fluid homeostasis and fetal and placental development.²¹ Cloned bovine fetuses with abnormal placentation are usually associated with excessive fluid accumulation in fetal sacs (hydrops syndrome). In this experimental model, cloned embryos with

hydrops syndrome surviving until midpregnancy have overgrowth of placentomes, fetal liver, and fetal kidney compared to normal pregnancies.²¹ In addition, organomegaly of offspring's liver, kidney, and heart is typically accompanied with polyhydramnios (in several mammalian species studied). Moreover, underlying mechanisms of fluid sac homeostasis are not fully understood in bovine pregnancy and further research is needed to elucidate cause and effect relationships.

Failure of proper organ development occurs in cloned calves that die shortly after birth. Using this experimental model, researchers have examined 8 developmentally important genes in 6 organs (heart, liver, spleen, lung, kidney, and brain). Of these developmental genes, kidney was the least affected organ associated with calf mortality, whereas heart was most affected by gene dysregulation compared to normal offspring.²² Of these developmental genes, vascular endothelial growth factor (VEGF) was upregulated in cloned offspring associated with early neonatal mortality. Vascular endothelial growth factor was implicated in stimulating vasculogenesis and angiogenesis to restore oxygen supply to tissues when blood circulation is inadequate. Appropriate concentrations of VEGF during organogenesis and early organ development are vital for proper establishment of fetal cardiovascular system. Downregulation of VEGF could decrease fetal organ angiogenesis, with fatal consequences to fetus (pregnancy wastage) or neonate (calf mortality); however, upregulation may result in organomegaly or fetal cardiac dysfunction, with similar fatal consequences to newborn.

Placental development and growth

Placenta has major role in fetal growth regulation. Ruminant placenta is morphologically classified as cotyledonary and histologically as syndesmochorial. In nonpregnant ruminants, caruncles are organized in 2 dorsal and 2 ventral rows that run length wise along uterine horns. Chorioallantois has flat surface that becomes irregular when it starts to cover caruncles, due to growth and expansion of conceptus within uterine lumen. This process is followed by recognition of cotyledons.²³ Caruncular-cotyledonary unit is called a placentome and is formed from growth and interdigitation of fetal villi and caruncular crypt adopting a convex shape.²⁴ Contact surface area is enhanced as cotyledon's finger-like projections enter crypts formed in caruncles. Placentomes vary in size; however, they are bigger at uterine horn base and decrease in size close to tip.²³ Placentome is the primary functional area of physiological exchanges between mother and fetus.

Efficiency of placental nutrient transport is directly related to uteroplacental blood flow.²⁵ All nutrients and wastes that are exchanged between maternal and fetal systems are transported via uteroplacenta.²⁵⁻²⁶ Establishment of functional fetal and uteroplacental circulations is one of the earliest events during embryonic/placental development.¹⁵⁻¹⁶ To support exponential increase in fetal growth during last half of pregnancy, proper growth and development of uteroplacental vascular bed must occur during first half of pregnancy.^{25,27} Understanding factors that impact uteroplacental blood flow will directly impact placental efficiency and thus fetal growth. However, despite much research in placental-fetal interactions area, regulators of placental growth and vascularization, including uteroplacental blood flow, are still largely unknown, particularly in cattle.

Our laboratory has been investigating blood perfusion and blood flow of reproductive tract using Doppler ultrasonography (applies Doppler Effect principle, named after Austrian physicist Christian Doppler; change in frequency of a wave for observer moving relative to source of respective wave). Example of this principle is perception of sound or differences in pitch one hears from stationary siren as person driving past the siren. Another example is that you are the stationary object and a car with a siren is driving past you. In this example, differences in pitch from moving siren will change as the car is driving towards you, the stationary object, and then away from you. In Doppler ultrasonography, stationary object is the transducer of ultrasound machine, which is detecting shift in frequency of red blood cells moving past stationary object.²⁸ Color can be assigned to normal B-mode, brightness gray scale image, by selecting color function of Doppler ultrasound machine. This will assign color based on directional flow (e.g. blood moving towards transducer probe is displayed in red and blood moving away from it is displayed in blue).

Good example of 2 vessels in close proximity and moving in opposite directions is illustrated in Figure 3A. Note the head and body of fetal sheep resting in amniotic sac at ~ day 40 of pregnancy. Top red arrow is pointing to umbilical cord, which is surrounded by color box and is showing a difference in directional blood flow from umbilical vein and umbilical artery. At this stage of pregnancy, umbilical cord is very small; however, it is still large enough to measure vessel diameter. Apart from color images, Doppler ultrasonography can estimate velocity of blood moving through a vessel. Figure 3B illustrates measurements taken while using Doppler mode. Sample gate cursor (green I) is placed on top of umbilical cord of a sheep fetus at day 90 of pregnancy (Figure 3B). Results are graphed in Figure 3C, where pulsatile umbilical artery cardiac cycle waveforms are visible above green x-axis line. Y-axis is detecting velocity (centimeters per second) of blood flow, whereas x-axis is depicting time in seconds. In addition, each pulsatile waveform of umbilical artery matches up with 1 fetal heart beat, enabling fetal heart rate calculation.

In addition to umbilical cord blood flow examination, our laboratory has used Doppler ultrasonography to examine uterine artery blood flow during mid to late pregnancy in cattle. Figure 3D illustrates a Doppler ultrasonography image of maternal uterine artery of a pregnant Holstein heifer ~ 180 days of pregnancy. Pulsatile cardiac cycles of uterine artery represent maternal heart rate. Moreover, these Doppler data allow calculation of velocity of blood flow during peak systolic and diastolic contractions of heart. Numbers on y-axis are in centimeters per second and are negative as red blood cells are moving away from transducer. In comparison, we also examined blood flow through hepatic portal vein of lactating dairy cows (Figure 3E). In this example, blood is flowing towards transducer, thereby giving a positive velocity on y-axis. In addition, nonpulsatile blood flowing through hepatic portal vein has an average velocity of ~ 50 centimeters per second. Data generated using Doppler ultrasonography are comparable to previous experiments estimating umbilical, uterine and hepatic blood flow with dye dilution techniques.^{7,8,18}

Data on development of bovine placentome capillary bed are limited. Cotyledonary growth progressively increases throughout pregnancy in cattle.^{29,30} Histological analysis of capillary bed development was performed in mid and late bovine pregnancies.³⁰ During this period, capillary area density, a measure related to blood flow, decreases ~ 30% in caruncular tissue, but increases ~ 186% in cotyledonary tissue. Also, number of capillaries increases ~ 150 and 80% in caruncular and cotyledonary tissue, respectively. Capillary surface density, a measurement related to nutrient exchange, increased in both caruncular and cotyledonary tissues (32 and 172%, respectively), whereas capillary size decreases 67% in caruncular tissue and increases 71% in cotyledonary tissue from mid to late pregnancy. Pattern of capillary development is very different between maternal and fetal portions of placentome, possibly due to energy demands of these independent tissues that share a similar function, delivery of nutrients to developing fetus. Placenta has fundamental role in supporting metabolic fetal demands. Although placental growth slows during last half of pregnancy, placental function increases dramatically to support exponential fetal growth rate.^{20,31} For example, in sheep and cattle, uterine blood flow increases ~ 3 - 4 fold from mid to late pregnancy.^{25,32-34} Relationship between uteroplacental blood flow and conceptus size throughout pregnancy is further defined below.

Cardiovascular adaptations during pregnancy

Physiologic state of dam is associated with significant but reversible alterations to metabolic demand and alterations to endocrine and cardiovascular systems. Maternal cardiovascular functional capacity changes dramatically during pregnancy, whereby systemic arterial blood pressure and vascular resistance decreases and cardiac output, heart rate, stroke volume and blood volume increase.³⁵ Although not all variables determined during bovine pregnancy, several mammalian species (including sheep) have decrease in mean arterial pressure in early pregnancy that persists throughout pregnancy. Moreover,

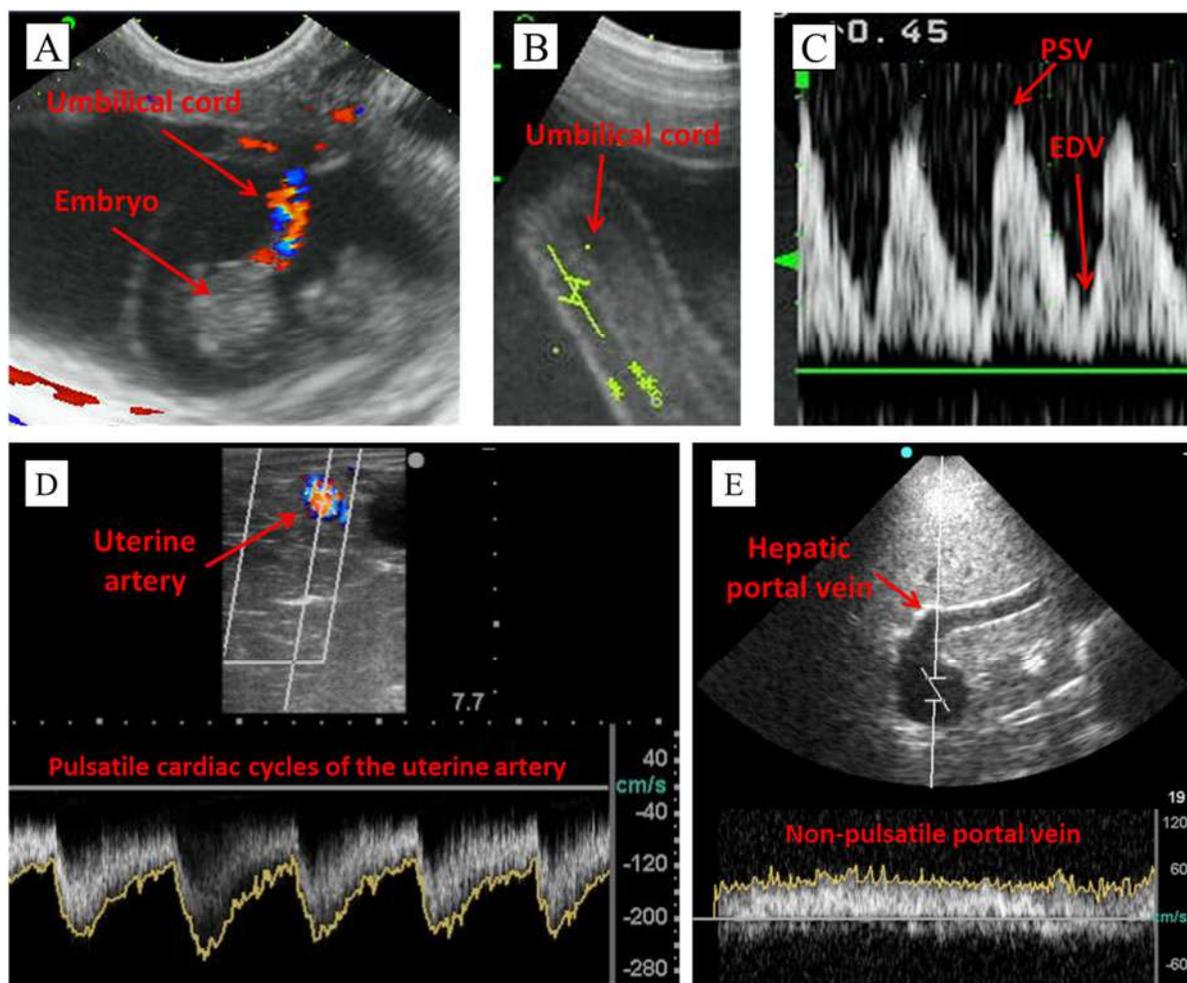


Figure 3. Doppler ultrasonography images of ovine umbilical cord at ~ day 40 (A) and day 90 (B) of pregnancy. Cardiac cycle waveforms of ovine day 90 umbilical artery (C) showing peak systolic blood velocity (PSV) and end diastolic blood velocity (EDV). Doppler ultrasonography image of bovine uterine artery and corresponding cardiac cycle waveforms (D) at day 180 of pregnancy. Doppler ultrasonography image of nonpulsatile bovine hepatic portal vein (E) of a lactating nonpregnant dairy cow.

decrease in arterial pressure (~ 5 - 10% decrease) is minor compared to ~ 20 - 30% decrease in total peripheral vascular resistance. Maternal cardiac output increases 30 - 40% in nonpregnant versus pregnant ruminants. Therefore, increase in cardiac output is associated with dramatic decline in systemic vascular resistance, allowing researchers to characterize pregnancy as a state of systemic vasodilation, resulting in profound increases in total systemic flows to all vascular beds. Most of these studies characterize this relationship by the equation; systemic vascular resistance = mean arterial pressure/cardiac output.

Using above equation, it is apparent that decrease in systemic vascular resistance during pregnancy and increase in cardiac output (total systemic blood flow) help to maintain arterial pressure. In addition, apart from this relationship, increases in blood volume and activation of renin-angiotensin system may also contribute to maintaining blood pressure during this physiological state of substantial vasodilation; however, limited data exists in cattle.³⁵ To determine specific contributions to increase in cardiac output occurring during pregnancy, we must first examine cardiac output equation, which states that: cardiac output = heart rate x stroke volume, whereby stroke volume equals volume of blood pumped from 1 ventricle of heart with each beat. In majority of mammalian species, heart rate increases by ~ 15% during pregnancy which does not fully explain 30 - 40% increase in cardiac output. Therefore, stroke volume may increase 30 - 35% during pregnancy and is one of the major contributors to this

increase in cardiac output. In sheep treated chronically with estrogen, induced increase in left ventricular heart dimensions and enlargement were similar to pregnancy; therefore changes to endocrine system during pregnancy may help mediate temporal changes to maternal cardiovascular function.³⁵

Rise in maternal cardiac output during pregnancy is also associated with increased plasma and blood volume in cows.¹⁸ Increased blood volume varies among species and depends on dam's nutritional status and singleton versus twin pregnancies. Bovine blood volume expands by 10 - 20% during pregnancy, whereas in litter-bearing species, blood volume may expand by 30 - 50% during pregnancy. With increase in plasma volume, dam must maintain a proper balance of water and electrolyte retention; therefore, similar to alterations in maternal arterial pressure, this increase in plasma volume will be integrated with the renin-angiotensin system, which can serve additional purpose as an extrinsic modulator of kidney function and urinary secretion. In addition, to dramatic changes in maternal cardiovascular system during pregnancy, it is even more noteworthy that most mammals return to nonpregnant levels of cardiovascular function within 2 - 5 weeks postpartum.¹⁸ Although lactating, high-producing nonpregnant dairy cattle have a substantial increase in cardiac output compared to nonpregnant and nonlactating counterparts, this redistribution of blood flow during transition period from uteroplacental vasculature towards mammary gland is still a phenomenal physiological feat enabling peak lactation shortly after parturition.

Several animal models of fetal programming were extensively studied in ewes,¹⁴ although extrapolation to cattle should be minimal, due to drastic differences in placental development between sheep and cattle (Figure 4). In ewe, placenta reaches its maximum size during first two-thirds of pregnancy, whereas ~ 90% of fetal growth occurs during last third of pregnancy.¹⁷ In contrast, bovine placenta continues to increase in size exponentially as pregnancy proceeds; however, bovine fetal growth is much greater compared to placental growth (Figure 4).²⁵ Placenta is involved in transporting nutrients and wastes between maternal and fetal circulations and altered placental function was associated with abnormalities in fetal development. Efficiency of placental nutrient transport is directly related to placental blood flow.^{25,26} Key factors affecting placental nutrient transfer capacity are size, nutrient transporter abundance, nutrient synthesis and metabolism, and hormone synthesis and metabolism.³⁶ Large increases in blood flow to reproductive tract are necessary to support both nutrient and waste exchange between mother and offspring. Several environmental factors negatively impact placental development and blood flow during pregnancy, all of which can hinder offspring health and vigor. Regulators of placental nutrient transport and uteroplacental blood flow are still largely unknown, with most research efforts focusing on rodent models, which are different from livestock species. Elucidating consequences of specific hormonal supplements on the continual plasticity of placental function will allow determination of important endogenous mediators of offspring growth and development.

Maternal nutrient restriction

Poor forage quality in grazing systems can negatively impact nutritional intake of beef cattle. Pregnant beef cows grazing poor forage can alter fetal growth during increased periods of developmental plasticity. Thus, provisions from environment can program these offspring to experience changes in mortality and morbidity rates, slowed postnatal growth, altered carcass weights, and meat quality characteristics.³⁷ Relationship between maternal nutritional plane during late pregnancy and calf mortality was examined as early as 1975, where maternal nutrient restriction for 100 days prepartum decreased calf birth weight by 7% and increased calf mortality rate by 10%, whereas an additional 20% of calves died between birth and weaning due to scours.³⁸ Direct effects of nutritional plane on offspring production characteristics are dependent on timing of insult and magnitude of nutrition deprivation in relation to fetal and placental development.³⁹ In dealing with timing, it is also important to consider separation of prenatal

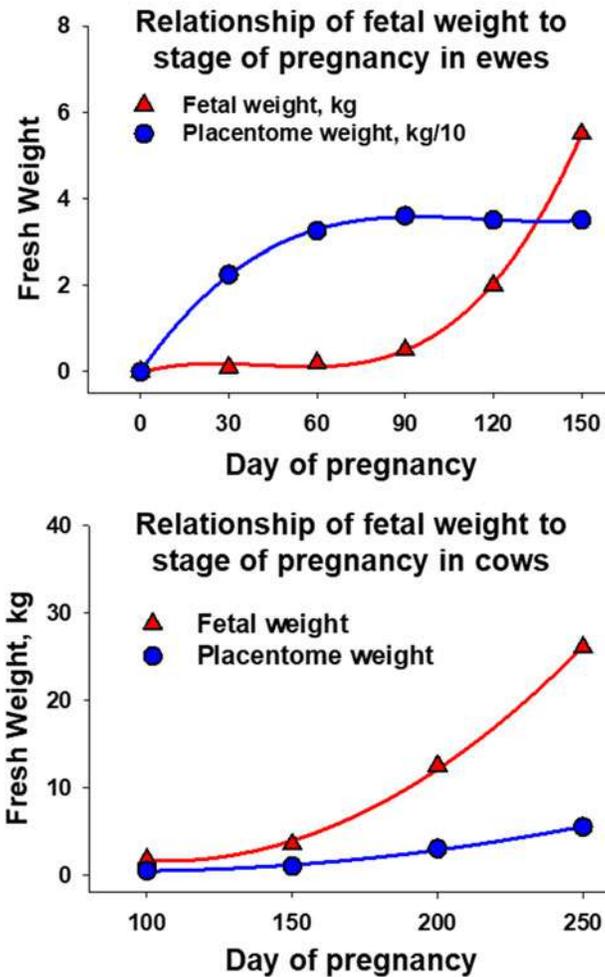


Figure 4. Fetal and placental growth in ewe and cow (redrawn).^{17,25}

versus postnatal maternal factors that may influence these developmental programming responses. For example, changes in meat quality of offspring born to dams experiencing late pregnancy maternal nutrient restriction may carry over into early lactation of this dam. Thus, researchers are prevented from identifying important time of maternal nutritional insult that led to negative offspring outcomes. This is vital when considering most economically feasible therapeutic interventions to mitigate negative developmental programming outcomes.

Adaptations of placenta during maternal nutrient restriction are incomplete and less is known about specific differences amongst breeds of cattle. Normal physiology such as pregnancy length, fetal growth, placental weight, even uterine blood flow can differ substantially between breeds of cattle.^{33,34} Therefore, magnitude of fetal programming is expected to be breed dependent and further research is needed to identify when and with what breeds should interventions be sought. In a recent study, we examined the effect of early to mid pregnancy nutrient restriction on uterine blood flow and fetal development in Brahman and Angus heifers.⁴⁰ Heifers were restricted to 60% of net energy requirements for pregnant cattle from days 50 - 180 of pregnancy. This early to mid pregnancy nutrient restriction decreased uterine artery blood flow and fetal weight at day 180 of pregnancy irrespective of heifer breed (Table 1). Moreover, efficiency of uterine artery blood flow relative to fetal weight was improved in nutrient-restricted versus adequate-fed dams (Table 1). A similar response was observed in Brahman heifers, irrespective of nutrient restriction, signifying a lesser amount of uterine blood flow needed to

grow similar weight fetus from a Brahman versus Angus dam (Table 1). A portion of these responses have been associated with increased placental efficiency in nutrient-restricted dams and Brahman dams.⁴⁰ Using a novel imaging technique to ascertain macroscopic blood vessel density of cotyledons (Figure 5), we observed significant increases in blood vessel density in Brahman versus Angus dams. As noted in the Figure 5 illustration, cotyledonary blood vessel density is heterogenous, with areas of high versus low vascularity within the same placentome. Moreover, nutrient restriction and decreased uterine artery blood flow increased cotyledonary blood vessel density, which we believe to be a compensatory mechanism within fetal membranes.⁴⁰ Angus and Brahman heifers subsets were allowed to calve and postnatal growth monitored through weaning (unpublished observations). Most postnatal measurements of growth were unaffected by maternal nutrient restriction from days 50 - 180 of pregnancy. However, heart girth increased in calves born to nutrient restricted versus adequate-fed dams, which may have in utero overcompensation of fetal growth when nutrient-restricted dams are re-alimented to adequate nutrition during late pregnancy.

Table 1. Uterine artery blood flow (BF) at day 175 of pregnancy and fetal flow at day 180 of pregnancy in nutrient-restricted (RES) or control (CON) fed Angus and Brahman heifers. Nutritional treatments were applied from days 50 to 180 of pregnancy. Data reported are main effect of nutritional plane and main effect of breed. Results adapted from.⁴⁰

Item	CON	RES	SEM	<u>P value</u> Trt
Nutritional treatment				
Uterine artery BF, liter/minute	5.04	2.33	0.73	0.035
Fetal weight, kg	9.56	7.35	0.61	0.047
Uterine artery BF by fetal weight, liter/minute/kg of fetus	0.52	0.29	0.07	0.058
Item	Angus	Brahman	SEM	<u>P value</u> Breed
Breed				
Uterine artery BF, liter/minute	4.66	2.71	0.76	0.119
Fetal weight, kg	8.04	8.88	0.61	0.388
Uterine artery BF by fetal weight, liter/minute/kg of fetus	0.53	0.28	0.07	0.054

Apart from nutritional management during pregnancy, we also examined heifer development practices and season on uterine artery blood flow during mid to late pregnancy.⁴¹ For example, beef producers opting for low-input forage-based replacement heifer management programs have lighter weights at breeding, with some heifers reaching only 50 - 55% of expected mature body weight at breeding versus a traditionally recommended target weight of 60 - 65% of expected mature body weight. We concluded that heifers developed on low-input management schemes until confirmation of pregnancy (days 30 - 45) had no compromise in uterine blood flow or calf birth weights compared to conventionally developed heifers. Moreover, volume of late pregnancy uterine artery blood flow relative to maternal body weight was significantly increased in low-input versus conventionally developed heifers, which may be a compensatory mechanism to safeguard fetal growth and development.⁴¹ In addition to low-input heifer development programs, we also examined effect of calving season on uterine artery blood flow, as differences in postpartum anestrus interval, conception rates, and weaning weights were reported between fall and spring calving herds.^{42,43} A portion of these responses could be programmed in utero via changes in nutrient and waste exchange between dam and fetus. In these initial studies, we observed an increase in uterine artery blood flow in the last third of pregnancy, consistent with exponential growth of fetus in spring- versus fall-calving heifers.⁴¹ Although cattle are considered nonseasonal breeders, seasonal

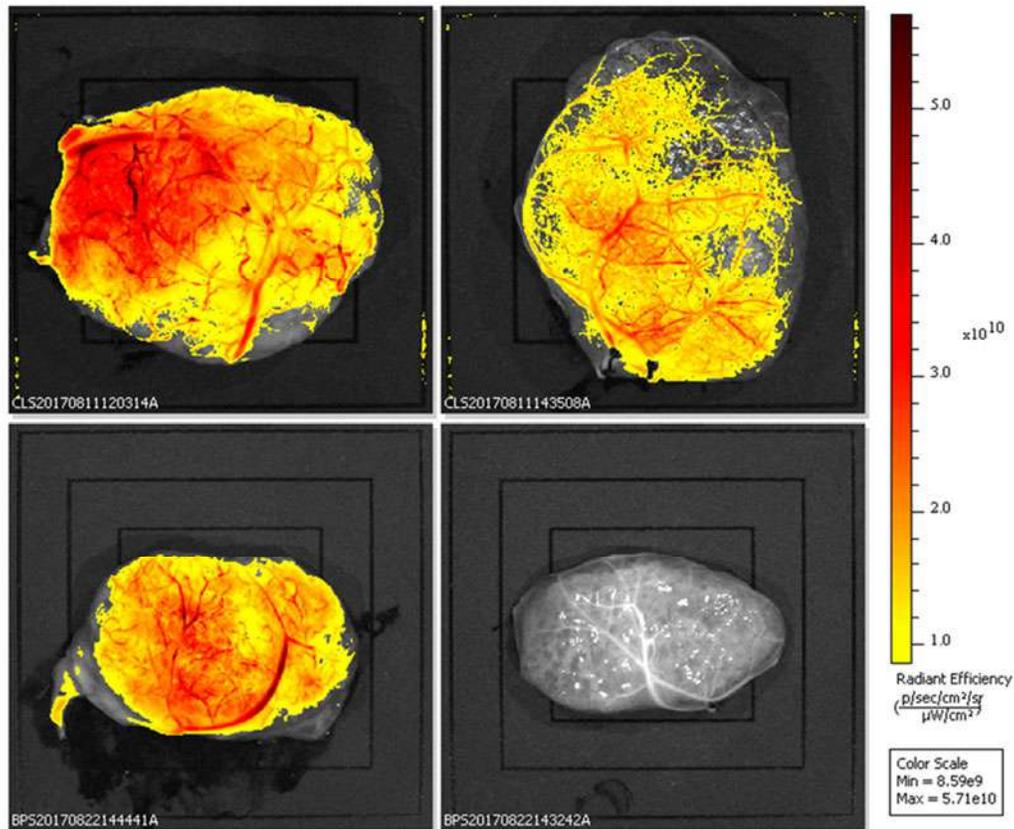


Figure 5. Fluorescent detection of macroscopic cotyledonary blood vessel density in placentomes collected at day 180 of pregnancy. Three representative images and one negative control placentome with cotyledonary surface up are shown.⁴⁰

changes in photoperiod, thermal stress, and nutrient availability can influence numerous performance and reproductive traits. Changes in hormone concentrations, as a result of photoperiod, may influence blood distribution to the reproductive tract, specifically changes in melatonin-modulated cardiovascular function.⁴⁴

Maternal melatonin supplementation

Amplitude of melatonin secretion has been associated with improved oxidative status and altered hormone metabolism in rats and sheep, and altered cardiovascular function in several mammalian species.⁴⁵⁻⁴⁷ In several studies, melatonin partially regulated blood pressure and blood flow.⁴⁸ Melatonin has both direct and indirect effects on the cardiovascular system and may cause either arterial vasodilation or vasoconstriction, depending on the origin of the blood vessel under investigation. Taking into account the above physiological responses, which can be partially altered by peripheral concentrations of melatonin, our research team examined effects of melatonin supplementation on uteroplacental development and functional capacity. Similar to other fetal programming models, our initial studies focused on pregnant ewe lambs. Using this sheep model of intrauterine growth restriction, we supplemented dietary melatonin as a potential therapeutic during mid to late pregnancy.⁴⁹ In our sheep model, ewes were supplemented with 5 mg of melatonin or no melatonin and allocated to receive 100% (adequate) or 60% (restricted) of nutrient requirements from days 50 - 130 of pregnancy. Using Doppler ultrasonography, we observed an increase in umbilical artery blood flow at day 130 in ewes supplemented with dietary melatonin, whereas uterine artery blood flow was unaffected by maternal melatonin supplementation.⁴⁹ At day 130, uterine artery blood flow decreased in nutrient-restricted versus adequately-fed ewes. Although melatonin supplementation failed to rescue fetal weight in restricted-fed ewes, we

observed similarities between fetal size and measurements of uterine and umbilical blood flow during mid to late pregnancy.

Recently, we examined uterine artery blood flow in Holstein heifers supplemented with 20 mg of dietary melatonin from days 190 - 262 of pregnancy.⁷ Uterine artery blood flow increased by 25% in melatonin-treated versus control heifers (Table 2). Surprisingly, calf birth weights were not different between treatments; however, calf body weight at 9 weeks of age increased in calves born to melatonin-supplemented dams versus control dams.⁸ Therefore, similar to other pregnancy models, an increase in uteroplacental blood flow during mid to late pregnancy is associated with alterations in postnatal offspring growth and development. This is apparent in the dairy heifer study, as calves were removed from dams, managed identically and fed a similar milk replacer and starter diet prior to weaning at 8 weeks of age. Therefore, postnatal maternal factors were removed from this dairy project, allowing us to propose direct fetal programming responses. Due to observed differences in dairy calf body weights, we replicated a similar experiment in beef cows. In this follow-up study, heifers and cows were assigned to 1 of 2 treatments: melatonin implants (MEL; n = 29) or no melatonin implant control (CON; n = 28) starting on day 180 of pregnancy and ending on day 270.⁹ As expected, uterine artery blood flow increased in commercial beef heifers and cows supplemented with melatonin during the last third of pregnancy (Table 2). Similar to the dairy heifer study, beef calf birth weights were not different; however, a 26 kg increase in weaning weight was observed in calves born to melatonin-supplemented versus control dams.⁹ Although results were similar to dairy study, it is important to note that postnatal maternal factors (e.g. postpartum cow health, colostrum composition, and lactational performance) could be contributing to increased weaning weights of calves born to melatonin-supplemented dams.

Table 2. Uterine artery blood flow (BF), calf birth weight, and calf weaning weight at 9 weeks of age from dairy heifers treated with (MEL) or without (CON) dietary melatonin from days 190 - 262 of pregnancy.^{7,8} Uterine artery BF, calf birth weight, and calf weaning weight at 28 weeks of age from beef cows treated with (MEL) or without (CON) dietary melatonin from days 180 - 270 of pregnancy.⁹

Item	CON	MEL	SE	P value
				Trt
Dairy heifers				
Uterine artery BF, liter/minute	5.73	7.16	0.35	0.02
Birth weight, kg	37.4	35.8	2.4	0.63
Weaning weight, kg	86.0	99.1	2.4	0.01
Item	CON	MEL	SE	P value
				Trt
Beef cows				
Uterine artery BF, liter/minute	5.76	7.87	0.57	0.01
Birth weight, kg	31.1	31.8	1.8	0.77
Weaning weight, kg	195.5	221.5	7.3	0.01

Taken together, results we observed in both cattle studies following melatonin supplementation allowed our research group to speculate on potential circadian alterations of reproductive tract during pregnancy. For example, rhythms generated from circulating concentrations of melatonin could mediate circadian rhythms in placenta and developing fetus during pregnancy. Potentially this could indicate a natural 24 hour rhythm in uterine artery blood flow, which may be influenced by exogenous melatonin supplementation. Therefore, from a livestock production standpoint, alterations in circadian rhythms during specific windows of pregnancy may lead to changes in offspring body composition; however, these fetal programming data are lacking. Nevertheless, the potential of establishing and/or disrupting these circadian rhythms during specific time points of pregnancy may alter production characteristics of offspring, which warrants further investigation.

Epigenetic mechanisms of fetal programming

Nearly every cell within an organism retains the entire genetic information or genome; however, cellular differentiation selectively programs different expression rates of genetic code. In contrast to earlier definitions of epigenesis, more contemporary usage of epigenetics refers to “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence”.⁵⁰ Therefore, epigenetic processes can program gene activity or expression patterns without altering the genetic code and differentiated adult cells have the capacity to retain epigenetic modifications from fetal development or even previous generations. Two primary mechanisms imparting epigenetic modifications to the genome are: 1) DNA methylation and 2) histone modifications.^{51,52} These modifications were implicated as the primary mechanism of developmental programming responses that may lead to later life noncommunicable diseases. An interesting proposal of epigenetic modifications relies on key nutritional cofactors associated with one-carbon metabolism, such as folate, vitamin B12, vitamin B5, choline, S-adenosyl methionine, betaine, and homocysteine.⁴ This proposal was shown in rodent studies, where dietary provisions of choline, folate, and vitamin B12 during pregnancy prevented DNA hypomethylating properties of bisphenol A, thereby altering gene expression and mitigating development of obesity in offspring.⁵³ Therefore, developmental origins of adult disease involve reprogramming the epigenome by environmental factors, e.g. maternal nutrition.⁵⁴

Postnatal outcomes

Animal models were developed and investigated to further our understanding of mechanisms underlying fetal origins of health and disease in humans.⁶ For example, maternal nutrient restriction during prenatal period is usually associated with low birth weights (intrauterine growth restriction) in offspring, as well as postnatal development of hypertension, obesity, and diabetes.⁵⁵ Lower birth weights were associated with increased postnatal morbidity and mortality,⁵⁶ poor postnatal growth, and decreased weaning weight in sheep.⁵⁷ However, a portion of these postnatal responses may be related to alterations in mammary gland development, which could lead to postnatal malnourishment and developmental programming responses occurring after the fetal period of development. Differences in birth weight can modestly predict carcass weight irrespective of dietary influences. Magnitude of growth restriction at birth in lambs, pigs, and steers was correlated with carcass yield leaving clear financial implications for intrauterine growth restriction in livestock species.⁵⁸ Specifically, in cattle, low birth weight was accompanied with slow postnatal growth, lower weaning weights and significant decreases in carcass weights at 30 months of age.⁵⁹ Some of these responses can be related to skeletal muscle development as improved nutritional plane during midpregnancy increased live weight and carcass weight and tenderness in crossbred steers.⁶⁰

Compromised pregnancies, via changes in maternal nutritional plane, were strongly associated with postnatal and later life changes to metabolic and endocrine functional capacity in offspring. Insulin sensitivity was a primary target of fetal programming as epidemiological evidence associated birth weight with an increased risk of developing type II diabetes.⁶¹ In an ovine maternal nutrient restriction model, malnourishment during late pregnancy decreased peripheral insulin sensitivity in young offspring.⁶² Similarly, changes to maternal nutritional plane (over or under-feeding) have been associated with increased lipid accumulation in liver of 1 year old sheep, as well as changes in hepatic fatty acid oxidation.^{63,64} Other endocrine changes in offspring born to compromised pregnancies were related to multiple hypothalamic-pituitary axes, e.g. those targeting thyroid and adrenal glands.⁶⁵ Since information related to adulthood and transgenerational consequences of fetal programming is limited, stringent environmental scenarios over several years are needed to fully relate these later life metabolic changes to fetal period treatments. In sheep, intergenerational programming of a metabolic syndrome phenotype was observed in grandsons and granddaughters of a maternal obesity model.⁶⁶ Moreover, these responses were sex specific, as granddaughters (F2 females) had greater insulin resistance compared to F2 males.⁶⁶

Although birth weight continues to be an indicator of potential adverse fetal programming events in offspring, it is important to note that birth weight provides limited information about body composition, morphometric size measurements, or individual organ size.⁶⁵ This is especially relevant in

early pregnancy maternal nutrient restriction studies, which usually do not alter birth weight or placental weight if dams are realimented to control diets prior to the exponential increase in fetal growth. Crossbred beef heifers subjected to maternal nutrient restriction from 11 days prior to AI through day 110 of pregnancy had increased concentrations of testosterone during nutrient restriction compared to control-fed heifers.¹⁰ On day 94 of pregnancy, prior to end of nutrient restriction, fetal size was not different between treatment groups; however, the diameter of aortic root increased in fetuses from nutrient restricted dams versus control. In this study, pregnancy length, placental weight, and birth weight were not affected by maternal nutrient restriction.¹⁰ Similarly, postnatal growth of offspring born to nutrient restricted dams was similar to controls until slaughter at 95 weeks of age. However, female offspring born to nutrient restricted dams had decreased ovarian reserves, enlarged aortas and increased blood pressure compared to their control counterparts.¹⁰ Therefore, in cattle, changes in maternal nutrition during first trimester of pregnancy may program female offspring reproductive characteristics and hypertension, which may be related to increased testosterone concentrations during pregnancy.¹⁰ Similar to these lack of changes in birth weight, we stimulated uterine artery blood flow via maternal melatonin supplementation in both dairy heifers and beef cows, with no significant impact on calf birth weight.^{7,9} However, postnatal growth and weaning weight increased in offspring of melatonin supplemented versus control dams.^{8,9}

Conclusion

Insufficiencies during pregnancy, resulting in reduced fetal growth and development, are detrimental to livestock production. We consistently observed positive associations with uterine and umbilical blood flow in sheep and cattle relative to fetal and postnatal offspring size. Doppler ultrasonography increased our understanding of blood flow and blood perfusion during important reproductive events, enabling producers to apply specific strategies to improve reproductive efficiency of livestock. Early to midpregnancy nutrient restriction increased placental efficiency in both Angus and Brahman heifers. However, late pregnancy nutrient restriction was associated with decreased birth weight, increased mortality and slowed postnatal growth of surviving offspring. Low input heifer development programs resulting in 50% of mature body weight at breeding did not negatively impact uterine artery blood flow and calf birth weight. Spring calving heifers had increased uterine blood flow compared to their fall-calving counterparts, which may be related to environmental differences and even hormonal changes with decreasing daytime length. Furthermore, we consistently identified increased umbilical and uterine blood flows in sheep and cattle during melatonin supplementation. Importance of this pathway in relation to development and transmission of 24 hour rhythms to offspring has not been elucidated in cattle. Negative insults during pregnancy were associated with dysregulation of metabolic and endocrine function in the postnatal period. Moreover, carcass yield and meat quality can be negatively affected by decreased or increased maternal nutrition during specific windows of pregnancy. Studies are needed to further expand these fetal programming responses related to adult offspring and potential transgenerational carryover.

Conflict of interest

There are no conflicts of interest to declare.

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Physiology and reproductive techniques for whitetail deer

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Abstract

Purpose is to briefly explain the physiology of whitetail deer and to cover reproductive management and artificial insemination techniques. Also, to cover some more advanced reproductive technology such as embryo transfer and in vitro fertilization.

Keywords: Cervid, laparoscopic, transcervical, embryo

Introduction

The commercial farming of whitetail deer is a relatively recent agricultural enterprise that came about to supply trophy animals to the hunting preserve industry. Whitetail deer range over most of North and Central America and are the most hunted big game species in North America. As many as 30 subspecies have been identified, with 16 residing in the US. The 2 that are economically important to deer farming are *Odocoileus virginianus borealis* and *texasus*, the northern whitetail and the Texas whitetail respectively. These subspecies typically grow the largest antlers and in the case of northern whitetail deer, there is also an increase in body size. To help advance the propagation of better genetics, artificial insemination has been employed to use different genetics in areas where the movement of live deer is not allowed. This has given rise to a very profitable industry.

Physiology

Whitetail deer are seasonal breeders with rut brought on by decreasing day lengths. As the days begin to shorten from midsummer into fall, there is an increase in hormones in both males and females of the species. In males, this triggers the hardening of the antlers with rubbing to remove the velvet, an increase in size of the secondary sex glands and testes, and beginning of spermatogenesis. More aggressive rutting behaviors start appearing and the bachelor groups start sparring for dominance and eventually break up before the main rut occurs. Female will phase from the anestrous period and after a number of silent ovulations, will come into the first behavioral estrous cycle. Any female not bred in the first cycle will continue to cycle at 21 to 29 day intervals until pregnancy occurs or increasing day lengths bring anestrous phase.¹ Estrous cycle of the doe is very similar to that of other ruminant species, in that luteolysis of corpus luteum from the previous ovulation decreases in blood progesterone concentrations and increases in estradiol concentrations produced by maturing follicles. A surge in luteinizing hormone from the pituitary gland in response to estradiol ultimately induces ovulation,² and the cycle starts over. Timing of rut varies from region to region, sometimes up to 8 weeks, but deer from a particular region will maintain the timing of rut even if moved to another location. This is important to remember when planning the timing of breeding, as the northern deer tend to rut in late October to mid-November, whereas Texas deer will rut in late November through December. This can be manipulated somewhat; however, timing too far outside their normal time frame will adversely affect conception rates. Whitetail does are polyovulatory, with most mature does producing 1.6 - 1.8 offspring on average. Yearling does are more likely to produce single fawns.

Reproductive management and artificial insemination

Whitetail deer as a group are exceptionally fertile and respond well to manipulation of the cycle during natural rut. Many of the protocols used in other small ruminant species translate well to whitetail does. The most commonly estrus synchronization currently used in the industry is a 14 day CIDR protocol. A 0.3 g CIDR (Eazi-Breed CIDR Sheep Inserts™, Zoetis Animal Health, Parsippany, NJ) is inserted into the vagina on day 0 and removed on day 14, at which time PMSG or eCG is given to synchronize ovulation. Breeding should occur 54 - 62 hours after CIDR removal. Alternately, CIDR can

be left for 14 days, then removed without PMSG treatment; however, breeding should be delayed a few hours (60 - 64 hours). In our experience, this protocol produces substantially lower conception rates compared to PMSG use. In both protocols, GnRH is given at artificial insemination (AI) to induce ovulation.

Artificial insemination can be accomplished in 1 of 2 ways (transcervical or laparoscopy assisted). Transcervical AI is less technical of the 2 approaches and equipment involved is less expensive than that used with laparoscopic AI, thus making it a popular choice for some producers. The does are most often bred without sedation after being secured in a handling chute. A vaginal speculum is inserted and the external os cervix is located. Any mucus should be removed. A goat or cattle insemination pipette is then introduced through the os and manipulated through the rings of cervix to deposit semen just cranial to cervix in the body of uterus. The downfall of the procedure is that the rings of the cervix are difficult to manipulate, and depositing semen in the cervix will result in decreased conception rates overall. Conception rates vary but usually will average around 65%. For optimal conception after transcervical AI, 12 - 15 million progressively motile sperm are recommended. Technician experience and patience will greatly influence the outcome.

Laparoscopic AI is more technical and the equipment used is much more expensive than transcervical AI. The minimum equipment needed is a quality rigid laparoscope of sufficient length to allow the technician to comfortably observe the uterus, a surgical or AI cart capable of holding the animal in dorsal recumbency with the head down at approximately a 45 degree angle, a good light source, 1 trochar and 2 cannulas, a source of filtered air or carbon dioxide for insufflation, aspics, and other disposable items. The procedure starts with a surgical prep and scrub of the caudal abdomen adjacent to the mammary glands. Two stab incisions are then made with a number 15 blade through the linea, approximately 10 cm below the junction of the udder. Another incision is made 6 - 8 cm off the midline on either side. Filtered air or carbon dioxide are then introduced to sufficiently insufflate the abdomen to allow the trochar and cannula to be introduced without damaging internal organs in this area. Once the cannulas are in place, the laparoscope is introduced, as well as the aspic containing the semen. Semen is deposited by placing the needle of the aspic through the wall of the uterine horn into the lumen. Ideally semen should be deposited in distal third of uterine horn ipsilateral to the dominant follicle, or split between both uterine horns if the ovaries are not observed. By depositing semen closer to oviducts, less semen can be used (~6 - 8 million progressively motile sperm) and conception rates are generally 10 - 15% better than transcervical AI. Once the procedure is mastered it only takes a few minutes to perform. Complications include rupture of the bladder, rumen, or uterus with introduction of the trochar. These ruptures should be repaired immediately to prevent peritonitis.

Advance reproductive technology

More advanced reproductive procedures are also being used in whitetail deer. Multiple ovulation embryo transfer (MOET) has become increasingly popular in recent years as protocols have successfully been adapted to whitetail does. Embryo recovery rates average around 5.5 embryos per doe. A multi injection program utilizing porcine derived FSH has been successfully adapted. The dosage varies based on a number of criteria, but FSH is administered every 12 hours for 4 days, beginning 3 days prior to CIDR removal. There is also a new recombinant FSH that recently became available through compounding that is showing promise. The procedure for recovery of the embryos is the same as for ewes and other small ruminant species. Animal is anesthetized and placed in dorsal recumbency with the head down at approximately 30 degrees. Uterus is exteriorized and an appropriately sized foley is placed in the base of the uterine horn near uterine body. Then a tom cat catheter or 18 gauge, IV catheter is placed in the lumen near the tip of the horn and flushed retrograde with 50 - 60 ml of flush media into an embryo concentration filter or petri dish. Incisions into the uterus should be stitched in an inverting pattern to avoid adhesion formation, and care should be taken with handling the uterus as whitetail are extremely prone to scar formation. The body wall and skin are then closed and appropriate antibiotics are given prior to reversal of the anesthetic. Once recovered, embryos can be transferred within a few hours fresh, or frozen for storage. For implanting embryos, a laparoscope is used to identify the corpus luteum. The

embryo is then placed into a tom cat catheter and the catheter is used to deposit the embryo into the lumen of the uterine horn ipsilateral to the CL. As deer are polyovulatory, 2 embryos are usually placed per recipient. Implantation rates of fresh embryos in whitetail is exceptionally high, often ~90%. Implantation rate for frozen embryos decreases to ~65%.

In vitro fertilization is a technology that has made tremendous advancements in recent years in other ruminant species. There is currently some work being done with whitetails; however, its use is not commonplace.

Conflict of interest

There are no conflicts of interest to declare.

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Urogenital surgery in camelids

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Abstract

Surgical interventions on the urogenital system are some of the most common procedures performed in veterinary practice. The present paper describes indications, techniques, possible complications and postoperative care of urogenital surgeries in camelids. Potential complications are discussed, based on retrospective analysis of clinical cases seen by the authors over the past 28 years of theriogenology practice. Although not part of the urogenital system, excision of the soft palate and rectal prolapse in camels are also discussed, as these disorders are related to reproductive activity in this species.

Keywords: Cesarean section, ovariectomy, laparoscopy, cryptorchidectomy, prolapse

Introduction

Urogenital surgeries are undoubtedly among the most commonly performed procedures in veterinary practice. They can be divided into 3 general categories: elective surgeries, surgeries to reestablish reproductive function, and emergency surgeries. Elective surgeries are primarily used to eliminate reproductive function (castration, ovariectomy), to prepare teaser animals (vasectomy), or to perform advanced reproductive techniques. Surgeries to restore or preserve normal reproductive function include, unilateral gonadectomy, and repair of the reproductive tract anatomy. Emergency surgeries in the female include cesarean section, laparotomy, and vaginal and uterine prolapse replacement. Emergency surgeries in the male, include primarily unilateral castration of an injured testis, preputial disorders, and urolithiasis. Excision of the soft palate is a frequent procedure in the dromedary camel. The present paper describes the indications, postoperative care and possible complications associated with reproductive surgeries based on the authors' experience. Some of the surgical techniques have been described elsewhere and are not detailed in this paper.

General consideration

The primary consideration prior to surgery is the approach to analgesia and anesthesia. These protocols are generally dictated by the type of surgery, species and the overall health of the patient. In the field, most elective surgeries are performed using a combination of chemical and physical restraint (Table 1). Deep sedation and field anesthesia may be obtained with higher doses of the same drugs. Heavier sedation and regional or local anesthesia is excellent for more involved surgeries, such as cryptorchidectomy and flank cesarean section. General anesthesia is mostly indicated in advanced techniques such as midline cesarean section, cystotomy, and laparoscopy. In the field, general anesthesia may be obtained with injectable anesthetics. In a hospital setting, general anesthesia is preferably induced with injectable anesthetics and maintained with gas (e.g. isoflurane in oxygen). Details are summarized in Table 1.

In nonemergency surgeries, feed should be withheld for 24 - 48 hours prior and water for 12 - 24 hours prior, depending on the size of the animal. Broad spectrum antibiotics and tetanus toxoid should be administered prior to surgery.

Postoperative pain management may include butorphanol tartrate (0.05 - 0.1 mg/kg IM), flunixin meglumine (2.2 mg/kg), morphine (0.1- 0.25 mg/kg IM, every 24 hours) or meloxicam (1 mg/kg, every 48 - 72 hours in alpacas and llamas, 0.5 mg/kg, orally every 48 - 72 hours in camels). It is extremely important to consider the degree of hydration and any potential compromise of kidney function, especially when using drugs known to be nephrotoxic.

Table 1. Common drug protocols used by the authors for sedation and anesthesia in camelids (doses are in mg/kg bodyweight, unless otherwise stated)

Drug	Alpacas and llamas	Camels	Remark
Sedation			
Acepromazine	0.15 IM/SQ	0.03 – 0.1 IM 0.01 - 0.02 IV	Urolithiasis
Butorphanol	0.05 - 0.2 IM	0.03 - 0.05 IV or IM	Standing castration
Detomidine	0.04 - 0.06 IM	0.02 - 0.05 IM	Restraint
Diazepam	0.5 - 0.2 IM	0.2 - 0.3 IM	Light to moderate sedation
Xylazine	0.1 - 0.5 IV 0.2 - 0.6 IM	0.1 - 0.25 IV 0.3 - 0.4 IM	Recumbent sedation at high doses
Anesthesia (Recumbency)			
Diazepam + Ketamine	D: 0.2 - 0.3 IM + K: 5 - 8 IM	D: 0.2 - 0.3 + K: 5 - 8 IM D: 0.1 - 0.2 + K: 3 -5 IV	
Xylazine + Ketamine	X: 0.22 - 0.44 IV + K: 2.2 - 2.5 IV X: 0.22 - 0.44 IM + K: 10 -15 IM 10 minutes later	X: 0.35 + K: 5 - 8 IM X: 0.25 + K: 3 - 5 IV	Anesthesia 30 - 60 minutes
Butorphanol + Ketamine + Xylazine	Alpacas: B: 0.046 + K: 4.6 + X: 0.46 IM Llamas: B: 0.037 + K: 3.7 + X: 0.37 IM	B: 0.3 + K: 3-4 + X: 0.2-0.3 IM	Anesthesia 30 - 40 minutes
Triple drip: Xylazine (1 mg/ml), Ketamine (1 - 2 mg/ml) in 5% guaifenesin	Induction: 0.6 - 1.1 ml/kg Maintenance: 2.2 mg/kg/hour	Induction: 1.1 ml/kg Maintenance: 2 ml/kg/hour	Maintenance CRI to effect
Propofol for induction	2 - 3.5 IV		Can be used for maintenance, but expensive

Urogenital surgeries in the female camelid

Cesarean section (hysterotomy)

Cesarean section in llamas and alpacas may be performed using a left flank or ventral midline approach.¹⁻³ The flank approach is the best choice under field conditions and for severely compromised patients. In camels, although a left ventrolateral approach has been described,⁴ the authors recommend only using a higher left flank approach because of risks of herniation using a ventrolateral approach.^{2,5}

For a flank approach, most females tolerate the surgery using local anesthesia with lidocaine administered in a line or inverted “L” block anesthesia (lidocaine diluted to 1% with isotonic bicarbonate or saline, with the total dose not to exceed 4.4 mg/kg) following sedation, caudal epidural and physical restraint in the sitting sternal (“cush”) position. The surgical procedure has been described by the authors and is similar to that performed in ruminants, except that the skin incision is oblique, extending from the angle formed by tuber coxae to the base (ventral aspect) of the last rib.¹⁻³ The incision line should be parallel to the direction of the quadriceps when the animal is sitting in the sternal position (Figure 1).

The subcutaneous muscle and fascia and the external oblique muscle are incised, whereas the internal oblique and transverse abdominal muscle may be gridded along the muscle fibers. The gravid uterine horn (always the left) is grasped around a fetal limb and gently exteriorized from the abdominal incision (Figure 2). A uterine incision is made over the limb at the greater curvature and the fetus is exteriorized (Figure 3). If the placenta is still attached, it should be left in place but peeled away from the uterus 2 - 4 cm along the entire uterine incision to avoid incorporating it into the uterine closure, resulting in subsequent dehiscence. Because of the type of placentation (epitheliochorial and microcotyledonary), mural bleeding is common and hemostasis is provided by over-sewing (“whipstitching”) the margins of



Figure 1. Flank approach for cesarean section in camelids with the patient restrained in sternal position. Note the oblique direction of the incision

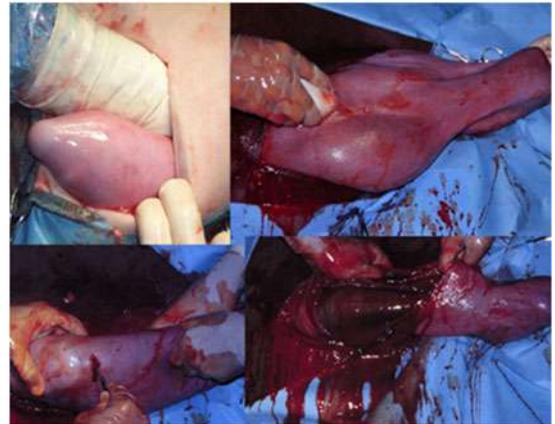


Figure 2. Flank approach for cesarean section in camelids. Exteriorization and incision of the uterus.



Figure 3. Flank approach for cesarean section in camelids. Uterine incision and delivery of the fetus.



Figure 4. Flank approach for cesarean section in camelids. Uterine suture using an inverting Utrecht pattern.



Figure 5. Flank approach for cesarean section in a llama. Skin suture



Figure 6. Midline approach for cesarean section in an alpaca with a 360° uterine torsion

the uterine incision in a continuous interlocking pattern using absorbable suture material. The uterine wall is closed in a Cushing, Utrecht, or Lambert pattern using absorbable suture material (Figure 4). If the uterus appears healthy, a single layer closure is sufficient; however, if there is compromise, a double layer closure is recommended. The uterine wall and abdominal cavity may be lavaged with a warm sterile saline solution containing antibiotics (penicillin G potassium 22,000 units/kg, ampicillin sodium 20 mg/kg, or ceftiofur sodium 1 mg/kg), and heparin (20 - 40 units/kg). Carboxymethyl cellulose (14 ml/kg, intraperitoneally) may be used to prevent postoperative adhesions. The abdominal cavity and skin are closed in the same manner as for ruminant cesarean section (Figure 5).

The midline celiotomy approach is performed in alpacas and llamas in dorsal recumbency under general anesthesia; this is the preferred approach if the uterus is compromised or has undergone torsion (Figure 6). A midline celiotomy incision (25 - 30 cm in alpaca and 35 - 40 cm in llamas) is made through the skin, subcutaneous fat, cutaneous trunci muscle and linea alba from ~ 4 cm cranial to the border of the mammary gland extending towards the umbilical scar. The uterus is identified by direct palpation and exteriorized. An incision is made through the uterine wall along the greater curvature. The fetus is removed and the umbilicus clamped and transected. Uterine closure is as described above. This technique can be used to resolve preterm uterine torsion without delivery of the fetus if it is still alive.⁶ If the fetus is dead or at term (dystocia due to uterine torsion), the fetus is delivered, then the torsion corrected. The linea alba is closed using absorbable suture material (No. 2 polyglycolic acid or No. 1 polydioxanone or polyglactin 910) in an appositional interrupted (horizontal mattress, cruciate) or continuous suture pattern. Closure of the skin may be achieved with staples, horizontal mattress suture pattern, Ford interlocking suture pattern or preferably where possible, a subcuticular suture pattern (No. 2-0 polyglactin 910 or polyglecaprone).

Postoperative care includes pain management for 3 - 5 days. Antimicrobial prophylaxis should be continued for 5 - 7 days, depending on the condition of the uterus and fetus at the time of surgery. Fluid therapy may be indicated in some cases. The dam should be monitored for clinical signs of postpartum metritis and toxemia. The placenta is generally expelled within a few hours of surgery if the cervix is open or 2 - 4 days if it was closed at the time of surgery. Oxytocin may be administered (20 IU IM in camels and 5 - 10 IU IM in south American camelids) every 4 hours during the first 24 hours postsurgery, if the cervix is open. Administration of cloprostenol IM (125 - 250 µg in alpacas, 250 µg in llamas, and 500 - 750 µg in camels) is recommended if the surgery was performed to remove a dead fetus due to uterine torsion.

Complications of cesarean section in camelids include retained fetal membranes, incisional infection, herniation, peritonitis, intestinal adhesions, and infertility (Table 2). However, these complications are minimal when the surgery is performed early in dystocia.⁷⁻⁹ The most common complications seen in camel cesarean sections, performed by the authors in the field, were incisional infections (myiasis) and herniation.

The rebreeding success rate is excellent (> 70%) following cesarean section, and most females will rebreed successfully 3 - 4 months postsurgery.⁶⁻⁹ The earliest successful rebreeding post cesarean section seen by the author is 45 days. The authors generally recommend at least 45 - 60 days of sexual rest, with a thorough prebreeding examination prior to any breeding following a cesarean section.

Ovariectomy/ovariohysterectomy

Ovariectomy is usually performed to prevent sexual activity and eliminate pregnancy risk or to remove a diseased organ (e.g. ovarian masses, ovariobursal adhesions).¹⁰ Ovariohysterectomy is rarely performed in camelids, but may be considered in cases of uterine or cervical masses and mucometra.¹¹ Ovariectomy or ovariohysterectomy should ideally be performed during the luteal phase of the cycle, so that the uterus is relaxed and complications associated with hemorrhage are reduced. Alternately, females should be given progesterone (progesterone in oil IM or intravaginal CIDR) for 7 - 10 days prior to surgery.

Table 2. Indication and outcome of cesarean sections in camelids

Reference	Campbell et al: 2013*	Miller et al: 2013*	Tibary et al: 2015**
No. females	24	31	76
Primarous (%)	45.8	-	-
Multiparous (%)	54.2	-	76
Uterine torsion (%)	61	38	0.5
Fetal maldisposition (%)	21.7		
Failure of cervical dilation (%)	8.7	17.5	81.65
Other (%)	8.6	22	
Dam survival (%)	91.2	86	92.1
Neonate survival (%)	46	59	81.6
Complications			
Retained fetal membranes (%)	8.3	88	15.7
Metritis (%)	-	-	2.9
Other complications (%)	-	-	20
Postsurgical fertility (%)	70	90.5	70.8

*Llamas and alpacas, **dromedary camel recipients in an embryo transfer center

In alpacas and llamas, ovariectomy may be performed using a parainguinal, ventral midline or flank approach. Laparoscopic and laparoscopic-assisted techniques are described in subsequent sections of the present paper. The choice of a particular technique depends on the age of the animal, the side of the ovary concerned (unilateral or bilateral), and the status of the ovary (normal versus abnormal). The flank approach is considered the best for camels.^{2,10} The technique is similar to the approach described for cesarean section, except that the incision is vertical and gridding is possible (Figure 7).

For a ventral midline approach, the anesthetized animal is placed in dorsal recumbency. A small (6 - 8 cm) incision is made on the ventral midline just cranial to the udder and continued into the abdominal cavity as described for cesarean section. The surgeon introduces 2 fingers into the abdominal cavity. The urinary bladder is identified, and the uterus is recognized in its dorsal aspect by following 1 of the horns to the uterine bifurcation. Vaginal manipulation with a sterile tube speculum or blunt ended rod by an assistant may help the surgeon locate the uterus. One uterine horn is grasped between the fingers and pulled towards the surgical incision. Both horns are exteriorized by gentle traction, followed by exteriorization of the ovaries.

For ovariectomy, the vascular pedicle of the ovary is isolated by passing forceps through the mesovarium, making sure to incorporate the ovarian artery and vein. A size 0 absorbable suture material is used to transfix the ovarian pedicle before transection. Large ovarian masses (e.g. granulosa theca cell tumor, teratoma) may require placement of overlapping transfixing sutures on the pedicle to prevent hemorrhage (Figure 7).

For hysterectomy or ovariohysterectomy, the mesometrium and broad ligament of each uterine horn are transected after ligation of blood vessels. Transfixation and circumferential ligatures, using absorbable suture, are placed proximal to the cervix. The surgeon should make sure to include the large uterine vessels located on each side. The uterus is transected at the level of the body between 2 hemostatic forceps. If the remaining portion of the uterine body is large, it should be closed with an inverting suture pattern before replacing it in the abdomen. Removal of 1 horn or portion of a uterine horn (a partial hysterectomy) is sometimes used in research settings or for treatment of pathology confined to one side of the abdomen. The technique is similar to a total hysterectomy, although a flank approach would be more possible. The vasculature supplying the ovary and ipsilateral horn is ligated and transected. Pregnancy is only possible if the left uterine horn with the ipsilateral ovary are normal and maintained. However, embryos may still be collected from females with right uterine horn and normal ipsilateral ovary.

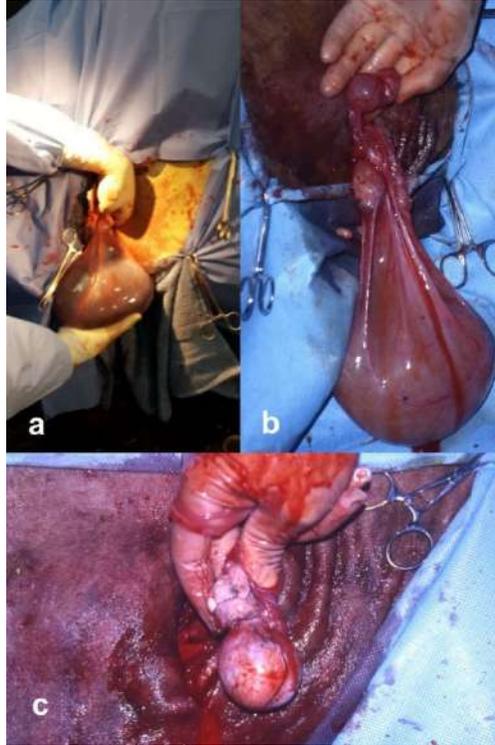


Figure 7. Paralumbar flank approach to ovariectomy in camels; a - b) removal of an ovary encapsulated with ovaribursal adhesions, c) removal of an ovarian teratoma

Postoperative care should include antimicrobial therapy and pain management. There are very limited risks of complication with ovariectomy. However, ovariohysterectomy may have a higher risk, due to intraoperative hemorrhage, adhesion formation and stump complications.

Laparoscopic techniques

Laparoscopy is widely used in reproductive research and for exploration of the abdomen and urogenital system abnormalities.¹²⁻¹⁴ The technique requires the use of a rigid laparoscope with a diameter of 6 - 10 mm and various lens angles, depending on the indication for the procedure. For most reproductive techniques in alpacas, we use a 6 mm diameter laparoscope with a 0 or 30° angle. This allows for minimal incisional size for portal placements. Standing laparoscopy may be used in llamas and camels using a 10 mm diameter laparoscope. For alpacas, laparoscopy is usually performed on the sedated or anesthetized animal in dorsal recumbency on a surgical table that can be tilted into Trendelenburg position. Animals should be fasted for at least 24 hours prior to the procedure to reduce forestomach compartment (C-1) fill and reduce risk of regurgitation.

Standing laparoscopy

Standing laparoscopy is performed mainly in llamas and camels for in situ observation of the genital organs or ovariectomy (Figure 8). The female is restrained in stocks and sedated with butorphanol tartrate. The left paralumbar fossa is prepared for aseptic surgery and 2% lidocaine is infused into the subcutaneous and muscular tissues in an inverted “L” pattern to desensitize the region (Figure 8).^{15,16}

A small (10 - 15 mm) skin incision is made in the craniodorsal portion of the paralumbar fossa caudal to the 12th rib (4.5 cm in llamas, 8 - 12 cm in camels) and ventral to the transverse processes of the lumbar vertebrae (8 cm in llamas and 12 - 15 cm in camels). This provides a portal for a 30° laparoscope, which is placed after penetrating the abdominal cavity through the abdominal musculature and peritoneum using an appropriate trocar. The abdomen is insufflated with CO₂ to a pressure of 10 mm Hg.



Figure 8. Standing laparoscopy in llamas and camels. Sites for laparoscope (1) and instrument portals (2 & 3)

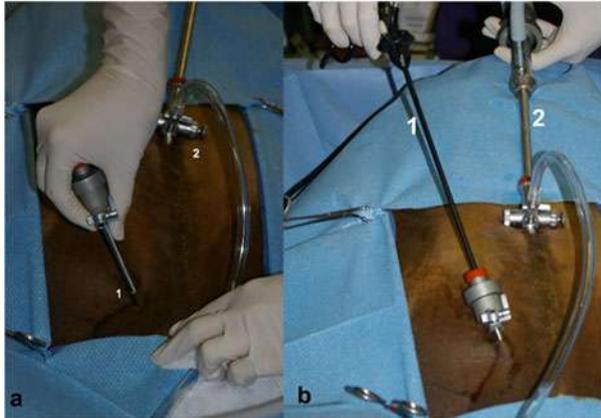


Figure 9. Ventral midline laparoscopy technique. Placement of the endoscope portals (1) and manipulation forceps portal (2).



Figure 10. Examples of abnormalities diagnosed by laparoscopic examination of the reproductive tract in alpacas. a) ovarian hypoplasia/dysgenesis, b) ovarian neoplasia, c) uterus unicornus, d) ovarian neoplasia, e) hydrosalpinx, f) adhesions

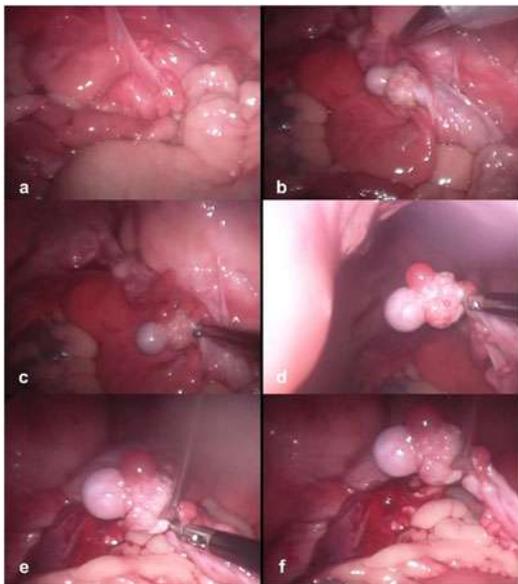


Figure 11. Laparoscopic-assisted ovariectomy in an alpaca. Ovary is dislodged from its bursa (a - b) then held with forceps from the pedicle and elevated to body wall (c - d). An allis forceps is introduced through a midline incision to hold the ovary and exteriorize it (e - f).

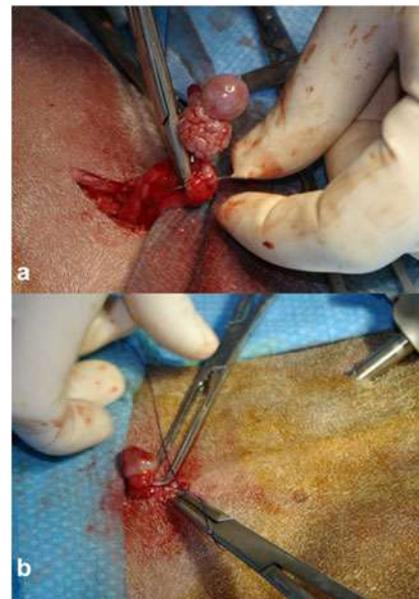


Figure 12. Laparoscopic-assisted ovariectomy in an alpaca. Pedicle of exteriorized ovary (a) is a transfixing ligature placed before excision (b).

Each ovary is identified by following the respective uterine horn from the uterine bifurcation to the tip of the uterine horn. This manipulation may be difficult if the urinary bladder is distended. Catheterization of the bladder with a Foley catheter prior to the procedure is helpful. Once the genital tract has been located and inspected, a second incision is made in the skin of the caudodorsal portion of the left paralumbar fossa caudal to the 12th rib (10 cm in llamas and 15 - 18 cm in camels) and ventral to the transverse process of the lumbar vertebrae (10 cm in llamas and 15 - 20 cm in camels). This incision will provide the portal for a manipulation instrument (grasping forceps) to be inserted through a cannula fitted with a sharp trocar. A small incision is then made in the skin of the caudoventral region of the left paralumbar fossa caudal to the 12th rib and ventral to the transverse processes of the lumbar vertebra to provide a portal for the introduction of a ligature guide holding a loop of size 0 polydioxanone. The ovary is grasped with the forceps through the suture loop. The ligature is pushed to the level of the ovarian pedicle and tightened around it. The ligature guide is removed, and scissors are introduced to cut the end of the ligature and transect the ovarian pedicle at the base of the ovary. The ovary is removed from the abdomen with the grasping forceps. All instruments are removed. It is important to ensure that excess gas is expelled prior to removal of the cannulas to avoid postoperative discomfort. To close portal incisions, use a single cruciate suture with 3-0 polyglyconate for the external oblique muscle and a single cruciate suture of 3-0 monofilament nylon for the skin.

Ventral abdominal approach

The anesthetized patient is placed in dorsal recumbency. The ventral abdomen is clipped and surgically prepared from the cranial edge of the mammary gland to the xiphoid. The laparoscope portal is made over the umbilical scar or a few centimeters caudal to it (Figure 9). A 10 - 15 mm skin incision (size depending on the diameter of the laparoscope) is made on the midline at the level of the umbilicus or 3 - 5 cm caudal to it, depending on the technique and length of the endoscope.^{1, 17-20} A controlled 2 - 3 mm stab incision is made on the linea alba and a teat canula or a verses needle is used to penetrate through the linea alba and peritoneum into the abdomen. Adequate penetration of the abdomen can be verified by injecting sterile saline through the canula. The abdominal cavity is insufflated with CO₂ to a partial pressure of 10 -15 mm of Hg. A cannula fitted with a pyramidal trocar is inserted at this level through the linea alba and peritoneum into the abdomen. The trocar is removed and replaced with a laparoscope. The pelvic inlet is identified by tilting the surgical table to 40 degrees to elevate the hind legs and displace the abdominal viscera cranially (Trendelenburg position).

For in situ examination of the reproductive tract, instruments portals are made lateral (left and right) to the midline midway between the scope portal and the caudal aspect of the fold of the flank. The uterus is located under the urinary bladder and each uterine horn is followed to its tip and elevated to allow visualization of the ipsilateral ovary. Atraumatic grasping forceps are used to manipulate the uterus and bladder for location and visualization of the ovaries (Figure 10).

For ovariectomy, the ovary is dislodged from the ovarian bursa by gentle manipulation until the mesovarium and proper ligament of the ovary are isolated (Figure 11). Hemostasis may be provided by two Hulka clips across the mesovarium or proper ovarian ligament, or a suture loop may be placed around these structures. The proper ligament is incised with scissors proximal to the clips or suture.^{17,18} Alternately, the ovary is grasped at the level of its pedicle after exteriorization from its bursa and elevated to the abdominal wall (Figure 11). An incision is made through the skin and linea alba as far caudal as possible, to avoid excessive tension on the ovary. A pair of Allis forceps is introduced through the incision into the abdomen and the ovary is grasped exteriorized (Figure 12). A transfixing suture is placed on the ovarian pedicle, using PDS-0, prior to excision of the ovary with scissors (Figure 12). The procedure is repeated on the other ovary.¹⁹ Closure of the external abdominal musculature (portal locations) and the linea alba is achieved using 2 polyglycolic acid in a cruciate and simple continuous pattern, respectively. The skin is closed with 2-0 polypropylene using a simple cruciate suture.

For laparoscopic ovariohysterectomy, the female is prepared in the same manner as for ventral midline laparoscopic ovariectomy. However, this technique requires more expertise in the handling of surgical instruments through laparoscopy. The procedure starts with ligation of the ovarian vessels. The

mesovarium is exposed and incised, after hemostasis has been established using a series of clips (Ligaclip[®]) inserted into the left portal. The broad ligament is transected close to the uterine horn caudal to the uterine body, avoiding the uterine artery. The same procedure is repeated on the other side. Once both uterine horns are freed, a ligature loop is introduced and passed around the uterine horns and ovaries all the way to the level of the uterine body, where it is tightened. A second ligature loop is placed in the same manner around the uterine body to provide adequate hemostasis. The uterus is transected, after placing a third loop along the portion of the uterus to be removed (to prevent loss of uterine content into the abdomen). The uterus and ovaries are removed from the abdominal cavity from 1 of the instrument portals after increasing its size.²⁰

Laparoscopic assisted ovum pickup

In preliminary trials by the authors, laparoscopic ovum pickup can be an alternative to laparotomy for alpacas.²¹ The technique is similar to that describe above for laparoscopic-assisted ovariectomy. Once the ovary is exteriorized from its bursa, an 18 gauge, 6 cm needle attached to an aspiration pump is introduced through the abdominal wall and follicles are aspirated. The recovery rate obtained (56.1%) was lower than the laparotomy technique.

Rectovaginal tear

Perineal lacerations are classified using the same system described for mares. The majority of cases observed by authors are third-degree and occasionally second-degree perineal lacerations. The occurrence of third-degree lacerations is common after severe dystocia, due to the small perineal body in these species (Figure 13).

Superficial lacerations can be repaired quickly under local anesthesia using a Caslick's procedure if the tear has already epithelialized. Second-degree lacerations require reconstruction of the perineal body, generally after sedation and epidural anesthesia. Repair of the third-degree perineal laceration and rectovaginal fistulas should be delayed until the tissue has granulated and epithelialized. Although some practitioners may perform this operation as early as 2 weeks after injury, it is the authors' preference to wait 4 - 8 weeks postpartum. Repair of third-degree laceration can be performed standing in camels. In llamas and alpacas, we prefer to have the patient heavily sedated or under general anesthesia in a sternal position (Figure 14). In sedated animals, epidural anesthesia is helpful, as rectal and vulvar tissues need to be retracted using suture or retractors. Repair techniques are similar to those described for the mare and consist of dissection of the rectal and vaginal walls, with the goal of creating a new separate rectovaginal shelf. The rectal and vaginal layers are closed with monofilament absorbable suture (2-0 or 3-0 PDS in llamas and alpacas, 0 or 1 PDS in camels). The perineal body is sutured separately, followed by skin suture.²²

Rectovaginal fistula

In the authors' experience, rectovaginal fistulas in camelids involve primarily the vestibular area (rectovestibular fistulas). They are better corrected by converting them to a complete rectovaginal tear prior to correction.

Chronic vaginal prolapse

Vaginal prolapse is relatively common in camelids, and particularly in heavy breeds of camels, in the last 2 months of pregnancy.²³ Predisposing factors include age (older females), parity, and body condition (obese or very thin females).³ In late-term animals, vaginal prolapse may be the first sign of a uterine torsion.

Prolapse of the entire vagina and exteriorization of the cervix is rare. Prolonged periods of prolapse lead to increased inflammation, resulting in severe necrosis of the vaginal mucosa. In some cases, ascending infectious placentitis is possible. In camels, myiasis of the prolapsed tissue is not uncommon (Figure 15). Chronic cases are at risk of abortion and/or rectal prolapse due to persistent tenesmus. Rectal and vaginal prolapse may be the only sign of uterine torsion, dystocia or abortion.



Figure 13. Third-degree perineal laceration following dystocia and fetotomy in a llama



Figure 14. Third-degree rectovaginal laceration repair in an alpaca. a - b) incision and dissection to separate the rectum from vaginal vestibule, c - d) Apposition of the flaps of tissue with sutures, ensuring that the rectal mucosa is apposed and the vestibular mucosa is everted into the vestibule. e - f) the perineal body is sutured using a simple interrupted pattern



Figure 15: Myosis complication of a chronic vaginal prolapse in a dromedary camel



Figure 16. Cercalge suture technique in an alpaca with recurrent vaginal prolapse due to rupture of the vestibule-vaginal sphincter. Left to right, the vaginal tissue is cleaned and replaced, a purse string suture is placed around the vestibular suture and a Caslick suture is placed to reduce the side of the vulva.



Figure 17. Uterine prolapse in a dromedary camel (left) and an alpaca (right)



Figure 18. Episio-epioplasty to repair a narrow vulva opening (incomplete atresia vulvi)

The prognosis for the life of the fetus and dam is relatively good if the condition is treated early. Prolapsed vaginal tissue is cleaned and replaced after sedation and epidural analgesia. In camels, the vaginal tissue is maintained in place by a Bühner suture around the vulva.²³ In alpacas and llamas, the vulva can be sutured with light umbilical tape, using a long postmortem needle in purse string or a shoelace suture pattern.²⁴ Sheep vaginal prolapse retainers have been used successfully to manage vaginal prolapse in alpacas.²⁵

Recurrent vaginal prolapse in postpartum alpacas and camels has been observed by the authors. In these cases, the vestibulovaginal sphincter may be compromised and the bladder may be entrapped in the prolapsed tissue. After replacement, the vestibular sphincter is held in place with an encircling suture using heavy resorbable suture material (Figure 16). Although the general recommendation is not to rebreed females with vaginal prolapse during pregnancy, there is no evidence for a hereditary nature of vaginal prolapse.

Uterine prolapse

Partial or total uterine prolapse can occur secondary to dystocia, abortion, manual removal of a retained placenta or excessive use of oxytocin (high dosage and frequency). Uterine prolapse is more common in camels than in llamas and alpacas, and is often associated with hypocalcemia, selenium deficiency and retained fetal membranes, particularly in dairy camels (Figure 17).^{2,26-28} Uterine prolapse generally occurs immediately (the first 60 minutes) after parturition or abortion.

Techniques for replacement are similar to those reported in cattle under sedation and epidural analgesia. The placenta is often easily peeled off and should be removed, if possible, prior to replacement of the uterus. The female is positioned in sternal recumbency, with the hind quarters slightly elevated. The uterus should be inspected for any lacerations or hemorrhage. The area of major risk for hemorrhage is located near the cervix where the uterine artery may be exposed. The uterus is cleaned with warm dilute povidone iodine solution before replacement. The vulva is sutured with a Bühner suture in camels or a shoelace pattern in alpacas and llamas. Uterine prolapse tend to recur if the uterine horns are not fully extended. Hysterectomy may be considered if the uterus has sustained severe damage; however the prognosis for survival in these cases is poor.^{27,29}

Vulvoplasty/episioplasty

Episioplasty is often considered in females with atresia vulvi or a recessed vulva (Figure 18). The surgery is straight forward and aims at extending the commissures of the vulva. However, owners should be warned about the possible hereditary nature of atresia vulvi and breeding these females should be discouraged.³⁰⁻³²

Vaginal adhesions

Vaginal adhesions are common complications of overt obstetrical manipulations or fetotomy in camelids.³ Laser surgery may be considered in some cases;³³ however adhesions often reform and in some cases, the surgery cannot be performed without risk of affecting the urethra (Figure 19).

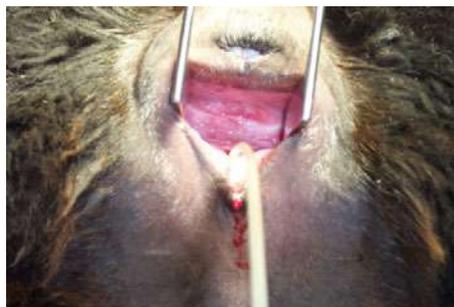


Figure 19. Vaginal adhesions in alpaca following fetotomy

Urogenital surgeries in the male camelid

Castration

Castration at an early age delays closure of the long-bone physes, resulting in tall, post-legged geldings and a predisposition to early onset of degenerative osteoarthritis or patellar luxation. The authors' recommended age for elective castration is 15 months for alpacas, 18 months for llamas and 2 years for camels. Precastration considerations include examination of the scrotum for testicular descent, lesions or adhesions. Presurgical administration of tetanus toxoid vaccination and antimicrobial therapy is recommended (Procaine penicillin G is generally given at 22,000 U/kg SC or IM before castration and daily for 3 days, or long acting ceftiofur 6.6 mg/kg SC once). Although the prescrotal technique has been described most practitioners use the scrotal approach.

The scrotal technique can be performed on the standing animal in llamas and camels, although lateral recumbency is recommended for alpacas.^{1,34} Standing castration is performed after sedation with butorphanol alone or combined with xylazine, followed by local scrotal and testicular infiltration of lidocaine. If castration is to be performed in lateral recumbency, a combination of xylazine, ketamine, and butorphanol is indicated. The scrotum is prepared for aseptic surgery and an incision is made along the most ventral aspect of the scrotum, with the scrotal skin taught by holding the testis firmly into the scrotum. The testis is exteriorized and held with a towel clamp while the testicular cord is gently stripped from fat and connective tissue, using a piece of gauze (Figure 20). The testis is removed using an emasculator (adult llamas) or after transfixation ligation of the spermatic cord with No. 0 (llamas, camels) or No. 2-0 (young llamas and alpacas) absorbable suture material. Unilateral castration is indicated in breeding males with a unilateral testicular disorder such as orchitis, trauma, or neoplasia (Figure 21).²⁹ The procedure is similar to conventional castration, except that a primary closure is recommended to promote fast healing and reduce the effects on the remaining testis. The vaginal tunic is closed with a simple interrupted pattern using absorbable suture material. Excess scrotal skin is removed and the subcutaneous tissue is apposed and closed with a simple continuous pattern. The skin is closed with a simple interrupted pattern using nonabsorbable suture material.

Postoperative care for castration includes antimicrobial therapy and confinement in a small pen for 24 hours. Topical antiseptic and fly spray are indicated under farm conditions. The animal should be observed for excessive bleeding or swelling, exudative discharge due to infection, and difficulty urinating. However, postcastration complications are rare (Table 3), and excessive bleeding can be managed by scrotal packing with gauze. Hemostatic agents are very helpful in controlling the hemorrhage in some cases. Under field conditions, the most common complication in camels is local wound infection and scirrhous cord development (Figure 22). It is important to inform the client that some males may continue displaying copulatory activity even after castration.

Cryptorchidectomy

Cryptorchidism is more frequent in alpaca and llamas than in camels.^{35,36} In 1 study in alpacas, there was 3% unilateral cryptorchidism in 792 animals. The left testis seems to be slightly more affected than the right testis (58.3 versus 41.7%).³⁷

Table 3. Complications following castration to remove normal descended testes in camelids

Complications	Llamas and alpacas*		Camels*	
	No.	%	No.	%
No complications	168	92.3	47	73.4
Anesthesia complication	1	0.5	-	-
Slight hemorrhage	4	2.2	-	-
Severe hemorrhage	1	0.5	-	-
Severe scrotal edema/preputial edema	8	4.4	15	23.4
Scirrhous cord	-	-	2	3.1
Total	182		64	

*All of these castrations were performed by veterinary student



Figure 20. Standing castration in dromedary camels

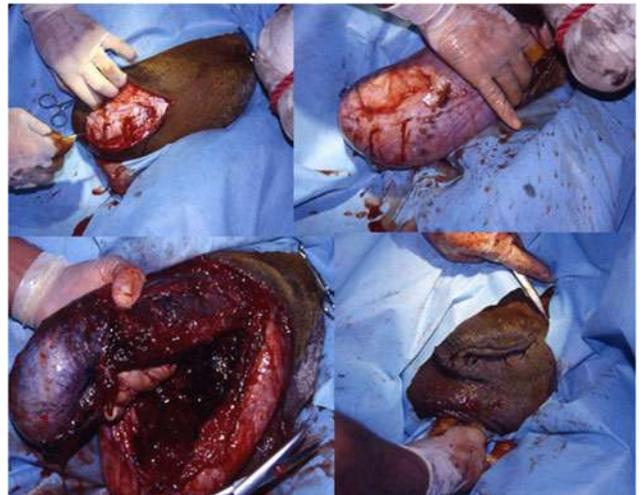


Figure 21. Unilateral castration with primary closure in a dromedary with a severe testicular hemorrhage and hematoma due to a biting injury



Figure 22. Scirrhous cord in a 12 year old male dromedary following castration

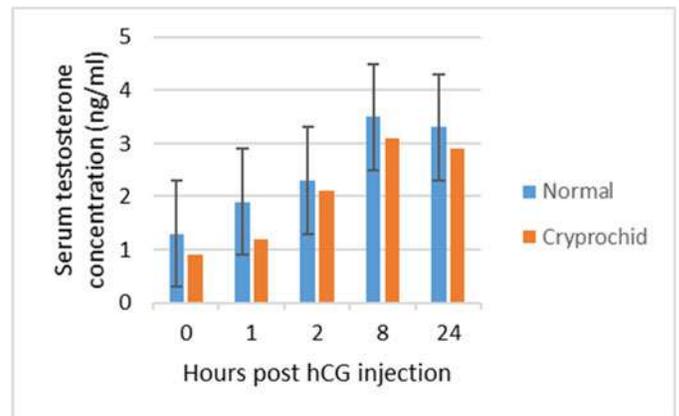


Figure 23. Serum testosterone concentrations in intact male alpacas (n = 8) and cryptorchid unilaterally castrated alpacas (n = 3) before and after hCG challenge

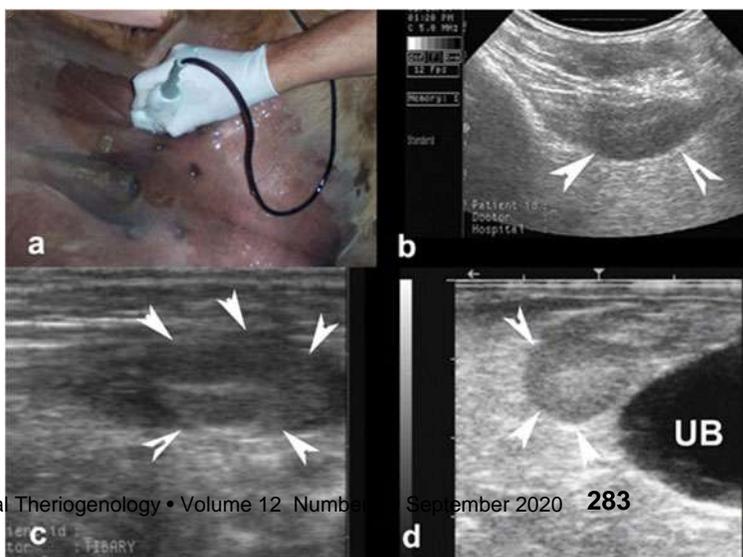


Figure 24. Ultrasonogram of a retained testis in a Bactrian camel (percutaneous inguinal, a - b) and alpaca (transrectal) (c - d, UB: urinary bladder)

Surgical approaches for cryptorchidectomy include flank, inguinal and parainguinal laparotomy⁴⁰ or laparoscopic assisted techniques.³⁶ For camels, the parainguinal approach is favored by the authors in camels.³⁵ The retained testis is often in proximity to the vaginal ring and may be retrieved using a spay hook. In some cases, direct exploration of the abdominal cavity is necessary.

For the parainguinal approach, the inguinal canal is located by palpation and a 1 cm skin incision is made medial to the caudal border of the external inguinal ring and extended cranially 3 - 4 cm. The incision is continued carefully through all tissue layers into the peritoneum. The abdominal cavity is entered using 2 fingers and the retained testis is identified by palpation of the area around the incision. The cryptorchid testis usually lies just lateral to the vaginal ring. Once identified, the testis is grasped and brought up to the incision site (Figure 25). The spermatic cord is ligated with 2-0 polyglactin 910 and resected. The internal abdominal oblique muscle is closed with 0 polyglactin 910 in a continuous pattern. The fascia of the external abdominal oblique muscle is closed with 1 polyglactin 910 in a simple interrupted or simple continuous pattern. To close the skin, a subcuticular closure in a simple continuous pattern is sufficient. Postoperative care should include nonsteroidal antiinflammatories and antimicrobials for 3 and 5 days, respectively.

Laparoscopy-assisted cryptorchidectomy offers the advantages of speed and reduced manipulation of the abdominal contents. The animal is placed in dorsal recumbency under general anesthesia and the ventral abdomen is prepared for surgery. The laparoscope portal may be placed over the umbilical scar. The intraabdominal testis is located and the second portal is placed laterally to the prepuce and used to introduce grasping forceps. The testis is grasped and elevated toward the inguinal area where a small parainguinal incision is made to exteriorize the testis (Figure 26).³⁶

A complete laparoscopic technique for cryptorchidectomy has been described and requires the use of 3 ports. The laparoscope portal is placed as described above and 2 instrument portals are placed on each side, ~ 5 cm from the prepuce. One portal is used to grasp the testis while the other is used to introduce laparoscopic, bipolar, electrocautery forceps. The spermatic cord is cauterized and transected and the testis is removed through the grasping instrument portal (Figure 27). The portal may need to be enlarged at this point. Portal sites are closed with a simple interrupted suture pattern. The skin is closed in cruciate pattern.³⁶

Vasectomy

Vasectomy is performed primarily for preparation of teaser males and for experimental studies where natural induction of ovulation (presence of β subunit of nerve growth factor in seminal plasma) without fertilization is desired. In alpacas and llamas, vasectomy may be performed via laparoscopy or a prescrotal technique under general anesthesia (Figure 28, 29).^{36,41} The inguinal region is clipped and surgically prepared. The spermatic cord is palpated lateral to the penis, caudal to the inguinal ring. A 2 cm skin incision is made over the spermatic cord and the vaginal tunic opened with a stab incision. The ductus deferens is isolated with hemostats. A 2 cm segment is isolated by blunt dissection and 2 ligatures of 2-0 polydioxanone are placed on each side of the segment prior to incision. The tunic is left open and the skin closed using 2-0 polydioxanone in a subcuticular pattern.^{42,43} Epididymectomy is an alternative to vasectomy, but is not favored by the authors (Figure 30).²⁹ Vasectomized males are sterile by 3 weeks after surgery.

Preputial prolapse, paraphimosis

Preputial lacerations are usually a consequence of masturbation behavior (breeding the ground or objects) or complications from foreign bodies within the prepuce. Hair rings around the penis are common in llamas and Suri alpacas. Presenting complaints may be similar to those for urolithiasis. In some cases, the only sign is preputial bloody or purulent discharge. Complications resulting in local adhesions and prevention of urination are possible.^{2,44} The penis and prepuce are evaluated under heavy sedation or general anesthesia. Early management of preputial and penile injuries should center on providing adequate protection of the traumatized tissue (and prevention of infection and complication with urine scalding ointment containing lanoline, castor oil and antibiotics).⁴⁵



Figure 25. Parainguinal approach to cryptorchidectomy in a Bactrian camel

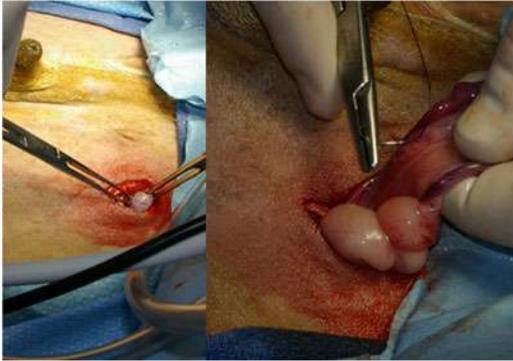


Figure 26. Laparoscopic-assisted cryptorchidectomy in a male alpaca



Figure 27. Laparoscopic cryptorchidectomy in a male llama

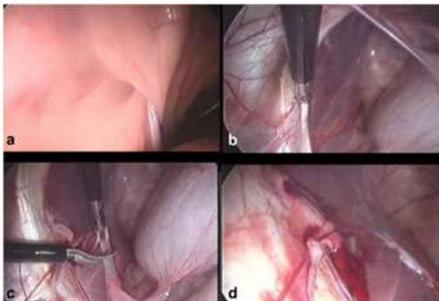


Figure 28. Laparoscopic vasectomy in a male alpaca. a) Identification of the vaginal ring, b - d) transection of the vas deferens



Figure 29. Inguinal vasectomy technique in camelids



Figure 30. Cauda epididymectomy in camel

Replacement of the healthy prolapsed preputial mucosa and its retention with a purse string suture is helpful in early cases (Figure 31). Daily cleaning of the sheath with saline and application of local antiinflammatory and antimicrobial ointment for 3 - 5 days will reduce the chance of further complications. Sutures may be removed after 7 - 10 days. Excessive preputial prolapse with slight necrosis requires circumferential resection and anastomosis of the prepuce. Long standing lesions have a poor prognosis, particularly if there is overt cellulitis and tissue necrosis (Figure 32). Paraphimosis is a common complication (Figure 33). Tissue necrosis is a common problem with these cases and may include the penis due to pressure ischemia. Surgical debridement and/or phallectomy may be required in these cases. In valuable racing camels, sand masturbation, the leading cause of preputial problems, is prevented by application of preputial rings (Figure 34).

Soft palate (dulla excision)

The soft palate (dulla) is exteriorized frequently during the rutting season in the dromedary.⁴⁶ Impaction of this diverticulum with food or foreign bodies results in entrapment of the tissue under the molars and traumatization during mastication (Figure 35). Severe edema and abscess formation are common complications, resulting in a permanent exteriorization and dysphagia.^{47,48} In rare cases, the soft palate is swollen but not exteriorized and blocks respiration, which may lead to asphyxiation.⁴⁹ Surgical management (i.e. palatotomy) is the best course of action. Excision of the prolapsed “dulla” is performed under heavy sedation or general anesthesia. The prolapsed tissue is excised after careful ligation of large vessels.⁴⁹⁻⁵² Postoperative care includes NSAIDs, antimicrobials and tetanus prophylaxis. Animals should be on soft food for 3 to 4 days after surgery.^{29,51} Palatotomy is often performed at a young age in racing camels to improve airflow during the breeding season.

Rectal prolapse

Rectal prolapse is common in obese male camels following excessive breeding, but can also be the results of other factors (e.g. diarrhea, excessive tenesmus, neoplasia, urolithiasis).^{53,54} It is classified in 3 degrees or types: Type 1 involves the rectal mucosa and submucosa, Type 2 involves the full thickness of the rectum and Type 3 may include intussusception of some small colon (Figure 36).⁵⁵ Types 1 and 2 are the most commonly encountered in breeding males and require sexual rest and sometimes surgical intervention. The rectal mucosa is replaced manually and retained with a purse string suture around the anal sphincter using a heavy nonabsorbable suture. The suture is left in place for 5 - 10 days. Mucosal resection and anastomosis are indicated if the mucosa is damaged. Amputation of the rectum is the last resort and carries a poor prognosis.^{53,54,56}

Cystotomy

Urolithiasis is relatively common in camelids. The etiology of urinary calculi in these species is not well understood but is suggested to be similar to that in other domestic ruminants.^{57,58} Clinical signs include persistent straining, odontoprisis, anorexia and ileus, anorexia, dribbling blood-tinged urine, and signs of abdominal discomfort.^{58,59} More severe clinical signs ensue in cases of complete blockage and rupture of the urethra or bladder. Fluid from abdominocentesis or the preputial swelling has increased creatinine concentration.⁵⁹ Increased serum urea nitrogen and creatinine concentrations suggest presence of uroperitoneum. Transcutaneous ultrasonography of the ventral abdomen may show subcutaneous free fluid and tissue edema in the case of urethral rupture and large volumes of free fluid in the abdominal cavity in the case of urinary bladder rupture. In the latter case, the urinary bladder may not be possible to image. Transrectal ultrasonography may reveal dilation of the pelvic urethra if the bladder is intact. Prognosis is grave in the presence of hydroureter and hydronephrosis.

Uroliths are often located in the distal penile urethra, ~ 7 - 12 cm from the penile orifice but may occasionally be found immediately proximal to the sigmoid flexure. Muscle relaxation and sedation may be obtained with acepromazine and diazepam (0.1 mg/kg slow IV). The urethral recess (diverticulum) at the ischial arch makes catheterization and retropulsion of uroliths impossible. The penis should be exteriorized and the tip of the glans penis (urethra) examined for presence of calculi (Figure 37).



Figure 31. Preputial mucosa prolapse and replacement in an alpaca



Figure 32. Preputial necrosis in an alpaca



Figure 33. Paraphimosis in a camel. a) before treatment, b - c) treatment with bandaging and emollient creams then suture in place, d) penis 7 months after treatment



Figure 34. Preputial ring for prevention of masturbation in dromedary camels



Figure 35. Traumatized permanently exteriorized soft palate in a dromedary (Courtesy of Dr. T. Mahendra⁵¹)



Figure 36. Rectal prolapse in a dromedary camel caused by excessive mating



Figure 37. Exteriorization of the penis and attempt to flush uroliths following catheterization of the urethra

Several surgical approaches have been attempted in alpacas and llamas, including urethrotomy, bladder marsupialization, tube cystotomy, and penile reefing.⁵⁸ Tube cystotomy technique is similar to that used in small ruminants on the anesthetized patient in dorsal recumbency using a paramedian approach. After exposure of the bladder, an incision is made into the bladder wall and the uroliths are removed followed by flushing of the urethra. A Foley catheter (12 - 18 Fr) is placed through a stab incision in the body wall of the inguinal region, inserted into the bladder and sutured in place using a purse string suture (Figure 38). The cystotomy incision is closed using an inverting suture pattern.

In camels, urethrotomy is the most common salvage procedure.⁶⁰ However, a case of successful management with tube cystotomy was recently described in a Bactrian camel.⁶¹ Overall, these surgeries carry a poor prognosis, particularly for reproduction.



Figure 38. Tube cystotomy in a llama

Conclusions

Surgical interventions on the urogenital systems are common in camelid practice. Veterinarians providing theriogenology services should be trained for a wide range of reproductive surgeries. Most of the surgical techniques are similar to those practiced in other large domestic animal species. However, case management should take into account anesthetic management and species anatomical differences. As data continue to accumulate on postoperative care and possible complications, veterinarians can provide better assessments when communicating with clients.

Conflict of interest

There are no conflicts of interest to declare.

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Postpartum factors and fertility in dairy cows

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Abstract

Normal reproductive function should return by 50 days postcalving and depends on complete uterine involution, clearance of uterine pathogens, and resumption of ovarian cycling. Delays in resumption of ovarian cycling beyond 35 days postcalving, failure to clear uterine pathogens, excessive adipose tissue mobilization and body condition loss, increased blood ketones, and decreased serum glucose are associated with decreased pregnancy to first insemination and increased pregnancy loss in cows that do conceive. Overall, health issues and delays in ovulation have an odds ratio of conception to first insemination of 0.54 - 0.39 relative to cows with early ovulation and no infectious or metabolic health problems. Embryonic losses are approximately 16.5% in cows with no health problems and 27% in cows with postcalving health problems. Significant proportions of cows with health problems challenge the ability of farm managers to achieve pregnancy rates that result in economic optimal reproductive performance.

Keywords: Postpartum cows, fertility, postpartum health

Reproductive Goals

Birth of a calf initiates lactation. Ideally, dairy cows would begin to produce milk at 22 - 24 months of age and calve every 11 - 13 months until replaced by a genetically superior female.¹ However, removal may occur for nongenetic purposes, such as for health problems, reproductive failure, or injury. Typically, cows remain in a herd for 2.5 - 3 lactations² until replaced by a younger female, but health and reproductive failure may influence the age at which a cow leaves the herd. Herd profitability is influenced by the dynamics of age at first calving, age of cow at replacement, annual rate of replacement, genetic intensity of replacement, and the annual frequency of animals recalving.¹ A successfully managed reproductive program controls the age at first calving (22 - 24 months), the period of annual recalving (calving interval, 11 - 13 months), the reason for herd removal (primarily low production), and the sale of female and male animals.

Annual recalving should occur every 11 - 13 months, with a rest period of 40 - 60 days between sequential lactations.¹ Since pregnancy is ~280 days in *Bos taurus* cows (range; 270 - 295 days), to maintain 11 - 13 month calving intervals requires that cows become pregnant 55 - 115 days postcalving. Since annual replacement (culling) rates are typically about 25 - 35% in a dairy herd, 65 - 70% of cows which calve should become pregnant within this interval, with new replacements maintaining the dairy population. Management practices that control insemination have a major influence on achieving this goal.¹⁻³ However, cow physiology also has a role determining if pregnancy will be achieved within economically optimal intervals. The economic success of herd reproductive programs depends on successful integration of management practices and cow physiology.^{1,4-5}

Pregnancy rate (PR) is a metric that captures management (insemination rate) and cow fertility (conception rate) in one variable.¹⁻³ It represents the proportion of nonpregnant (open) cows that become pregnant every 21 days.^{2,3} The average estrous cycle of the cow, 21 days (range; 18 - 24 days), delineates the time period for determining rates of insemination and conception. For this paper, conception will be defined as cows diagnosed as pregnant between 30 - 45 days postinsemination, relative to a particular time period of insemination. True, physiologic conception (fertilization of an ovulated oocyte) are close to 85 - 90% in dairy cows but are not observable on dairy farms.⁵ Embryonic loss of fertilized oocytes prior to 35 days are high (40 - 60% of fertilized oocytes) and still significant up to 45 days postinsemination (10 - 15% of pregnancies from 28 to 45 days).⁵

Pregnancy rate depends on insemination rate (IR) and conception rate (CR). Insemination rate is calculated as nonpregnant (open) cows inseminated within a 21-day period, divided by the proportion of

nonpregnant cows available to inseminate. Conception rate is calculated as the proportion of cows diagnosed as pregnant, divided by the total cows inseminated within the concurrent 21-day interval. Every 21 days following the voluntary waiting period (VWP), the proportion of open cows that become pregnant are determined by the PR. Pregnancy rate is the combination of IR (heat detection rate) times CR. Survival curves of pregnancy with days postcalving describe overall PR for a herd.

For optimal economic returns, PR needs to be $\geq 25\%$ (Figure 1). Pregnancy rates $< 25\%$ have 6-fold economic loss compared to $PR \geq 25\%$ (Figure 1). To achieve a $PR \geq 25\%$, insemination rates need to be $\geq 70\%$, and CR needs to be at least $\geq 35\%$. Management controls IR. Cow fertility is the major controller of CR. This paper will examine cow factors influencing PR. A companion paper will examine management factors influencing insemination rates.

Marginal Change in Value: Slope changes at a PR of 25%

The slope of the curve is the marginal change in value with PR
5.9x the rate of loss when $PR < 25\%$ compared to $PR > 25\%$
Goal is for $PR \geq 25\%$

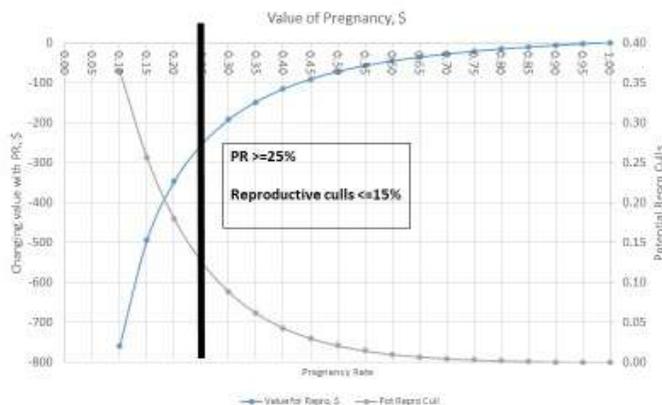


Figure 1. Pregnancy rate (PR) on the X-axis and change in economic value (losses) relative to a pregnancy rate of 1.00 on the left Y-axis, with potential reproductive culls on the right Y-axis. Cows that become pregnant in the first 21 days after the voluntary waiting period have a pregnancy rate of 1.00. With each successive 21 days, PR declines proportionally. The distribution of cows within each 21-day window determines the herd overall PR. Cows not pregnant by 150 days, typical for most herds, are at risk for culling, the proportion on the right Y-axis. Reproductive losses are much greater when the mean herd PR is below 25% and potential reproductive culls increase.

Parturient cow

Ideally, by 50 days postpartum, a cow is fully capable of becoming pregnant and carrying a pregnancy to term.⁶ This requires a fully regenerated and repaired uterus and resumption of estrous cycles prior to 50 days postpartum.⁶⁻⁸ During this period, milk production increases to peak yield, prioritizing nutrient demands for glucose, amino acids, and minerals.⁹ Peak milk production occurs by 30 - 50 days postcalving, yet feed intake will not peak until 100 - 120 days postcalving. This creates negative balances in energy, protein, and minerals, especially in the first 2 weeks postcalving.¹⁰⁻¹⁷ Positive balances will not be realized until 8 weeks postcalving, as feed intake increases and milk production declines from peak.¹⁶ At calving, homeorhetic signals coordinate nutrient flows to support lactation through alterations in tissue utilization of glucose, increased gluconeogenesis in the liver, mobilization of fatty acids from adipose tissue, and mineral mobilization from bone.^{4,9,15} Central control centers in the arcuate nucleus of the hypothalamus sense the degree of disparity in nutrient balance and turn on, dampen, or shut down hypothalamic centers that control GnRH release, impacting the initiation of ovarian function.^{14,18} Endocrine and metabolic signals influence central control centers that determine when reproductive function will commence. Endocrine and metabolic factors in the week prior to calving and in the first several weeks postcalving influence resumption of ovarian cycling and fertility at first insemination.^{15,19}

During the first weeks postcalving, homeostatic processes may be disrupted, resulting in ill health in the postpartum period (milk fever, ketosis, fatty liver, displaced abomasum); furthermore, infectious diseases (metritis, mastitis) further impact the reproductive axis and influence ovulation and future fertility (Table 1).¹⁹⁻²¹ In addition, elevations in nonesterified fatty acids (NEFA), serum beta hydroxy butyric acid (BHBA), and reductions in calcium have been associated with increased days to first ovulation, lower conception at first insemination, and reduced pregnancy rates.²²⁻²⁶ Energy balance, metabolic stress, and infectious disease combine to reduce fertility in the postpartum cow.¹⁹

Table 1. Postpartum conditions and fertility in dairy cows measured as the odds of conceiving at first insemination (OR, odds ratio)¹.

Item ⁴	Signalment	Action Level (%) ²	OR FSTCR ³
Dystocia	Calving difficulty	> 10	0.80,0.75
Twins		> 5	
Die	Death losses < 30 days	> 5	
Culling first 60 days		> 10	
Milk Fever	Hypocalcemia	> 2	0.85
Retained Placenta	> 24 hours	> 8	0.68
Puerperal Metritis	Fever > 39.7°C, off feed	> 10	0.56,0.64,0.43,0.37
Chronic Metritis	Purulent discharge > 30d	> 20	0.57, 0.68, 0.34, 0.24
			0.381, 0.71, 0.70, 0.67
Subclinical Endometritis	Proportion of neutrophils in uterine cytology	> 20	<1.00
Ketosis	b-OH butyrate > 1 mmol/l	> 10	0.69, 0.50, 0.79
Displaced abomasum		> 3	
Anestrus			0.47, 0.38, 0.42
Ovulatory dysfunction	> 40 DIM first ovulation	> 20	
Body condition loss	30 DIM \geq ¼ score loss	> 15	0.545,0.689
Body condition postpartum	score < 2.5 at 30 DIM	> 15	0.650
One clinical disease	in first 30 DIM	> 30	0.52, 0.79
Two or more clinical disease	in first 30 DIM	> 10	0.57

¹OR = odds ratio,

²Action Level = proportion of animals with the condition divided by the number calving

³FSTCR = first service conception rate

⁴Dystocia = based on calving difficulty score of 2 or greater

⁴DIM = days in milk

⁴Body condition based on a 5 point scale, 1 being emaciated, 2 thin, 3 adequate, 4 fat, 5 obese

Fertility is the outcome of multiple systems acting in concert in the postpartum dairy cow.^{18,19} The immune, metabolic, endocrine, and gastrointestinal systems undergo adaptations following calving to coordinate lactation, uterine repair, feed intake and resumption of reproductive cycling.^{15,19} Excessive inflammation and infection, increased metabolic disparity between nutrient intake and output in milk, and gastrointestinal upset can disrupt the coordination of nutrient flows and lead to impairment of health and fertility.^{20,21,27} Homeorhetic controls are achieved through changes in somatomedins postcalving.^{2,4,28,29} Serum insulin and insulin growth factor 1 (IGF1) decrease, whereas serum growth hormone, prolactin, parathyroid hormone, and glucocorticoids increase.^{4,15,18,28,29} Immediately postcalving, serum glucose declines and serum NEFA increases, released from adipose tissue.^{4,15,30} Amino acids are released from skeletal muscle. Plasma calcium declines precipitously on the day of calving and returns to homeostatic concentrations over next 4 days. Liver gluconeogenesis increases to meet the demand for glucose by the mammary gland using propionate absorbed from the rumen, and lactate and amino acids released from skeletal muscle.¹⁷

Serum BHBA increases due to incomplete beta oxidation of NEFA in the liver and peripheral tissues. Liver triglycerides increase due to incomplete incorporation of NEFA into triglycerides for export from the liver as low-density lipoproteins. Reduced serum insulin and increased insulin resistance in

insulin-sensitive tissues direct glucose to the mammary gland and reduce glucose availability to muscle and adipose tissue. Increases in inflammatory cytokines from uterine endothelial cells, macrophages, and neutrophils in response to uterine infection stimulate the liver to produce acute phase proteins, such as haptoglobin, serum amyloid A, and ceruloplasmin, and decrease synthesis of negative acute phase proteins, such as albumin, and vitamin and hormone carriers.^{20,21,27} Reactive oxidation compounds increase, increasing the demand for antioxidant protection.³¹

Decreases in plasma insulin, IGF1, calcium and glucose combined with increases in NEFA, BHBA, and inflammatory compounds influence the hypothalamic-pituitary-ovarian axis and may disrupt ovarian follicular growth, steroidogenesis, and ovulation.^{4,18,29} In addition, failure to clear pathogenic bacteria from the uterus further impacts the ability of the cow to establish and maintain pregnancy.^{7,8,32,33} Physical conditions at calving, such as dystocia, twin births, and uterine prolapse create risk factors for reduced fertility.³⁴⁻³⁸ Disease in the postpartum period, the first 30 days in milk, such as metritis, ketosis, milk fever, displaced abomasum, mastitis, and lameness, increase the risk for low fertility.³⁴⁻⁴² Increased negative energy balance, calving problems, uterine disease and metabolic disease disrupt uterine involution, ovarian activity, and pregnancy maintenance.

Cow factors influencing fertility

Postpartum cows experience multiple challenges postcalving. Parturition is an inflammatory process and inflammatory cytokines increase, influencing hepatic function and exerting central influences on feed intake and reproductive control.^{20,21,27} Uterine bacterial contamination is present in over 90% of cows in the first 3 weeks postcalving, further contributing to inflammatory challenges.^{7,8} Parturition is associated with depression in innate immunity, increasing the risk of bacterial contamination becoming a significant infection.^{7,8} Specific bacteria have a greater risk of causing uterine pathology.^{7,8} Uterine infection may present as an acute puerperal metritis observed in the first 2 weeks postcalving. Puerperal metritis is characterized by a fetid, watery vaginal discharge, fever > 39.5°C, and a depressed, anorexic cow. Failure to clear pathogenic bacteria by 3 - 4 weeks postcalving can result in a chronic endometritis or subclinical endometritis associated with purulent to mucopurulent vaginal discharge or an increased presence of polymorphonuclear cells in uterine lavages.^{7,8,32,33}

Superimposed on inflammatory and infectious challenges are metabolic challenges associated with the negative energy balance postcalving.⁴ In the first 3 weeks postcalving, significant elevations in serum NEFA (> 0.70 mEq/L), and BHBA (> 10 mg/dl), and reduced serum concentrations of glucose are associated with reductions in conception at first AI.²²⁻²⁶ Serum glucose is important in coordinating insulin and IGF1 in the early postpartum period and low serum glucose in the first week postcalving is associated with lower FSTCR.⁴ In addition elevations of NEFA in the week prior to calving are associated with reduction in FSTCR. Elevations in serum NEFA, BHBA and decreases in serum calcium and glucose have been associated with reduction in FSTCR in cows with no apparent clinical conditions postcalving.^{22-26,30,40} Metabolic stresses late in pregnancy and early postcalving influence FSTCR.

Metabolic influences in early postpartum could alter follicular and ovum development which would reduce fertility at first insemination.⁴³ Changes in body condition (BC) postpartum seem to support this hypothesis, as cows with more extreme condition loss have lower fertility.⁴⁴⁻⁴⁶ Similarly, cows with more body weight loss have fewer fertilized oocytes and transferable embryos.⁴⁷ Cows that experience more negative energy balance, more condition and body weight loss, and have greater serum NEFA in the first week postcalving have lower fertility at first insemination. Health problems postpartum exacerbate energy balance, as feed intake is often depressed, which may compound problems with fertility at first insemination.

Conception at first insemination postcalving for cows with various reproductive disorders is summarized (Table 2). Postpartum disorders have an odds ratio (OR) < 1, indicating a reduction in CR to first insemination. Cows with no health problems postcalving and that ovulate prior to first insemination have an odds ratio of first service of 1; they are the sentinel fertility group in a herd.

Table 2. Odds ratio of pregnancy at first insemination from various references

Author	Group	Year	Herds	Cows	OR	SEM
Fourichon et al:		1999	Meta-analysis		studies used varied with condition	
	Physical Dystocia				0.802	0.025
	Physical Still birth				0.739	0.081
	Metabolic Milk Fever				0.852	0.483
	Metabolic Retained Placenta				0.681	0.014
	Infectious Metritis				0.563	0.036
	Metabolic Cystic Ovaries				0.786	0.058
	Metabolic Anestrus				0.471	0.022
	Metabolic Ketosis				0.754	0.256
	Metabolic Displaced Abomasum				1.000	
	Physical Lameness				0.852	0.123
Ribeiro et al:		2013	2	957		
	Physical Dystocia				0.460	0.128
	Infectious Metritis				0.370	0.122
	Infectious Endometritis				0.680	0.138
	Infectious Mastitis				0.700	0.140
	Infectious Pneumonia				0.490	0.255
	Metabolic Digestive Problem				0.220	0.122
	Physical Lameness				0.510	0.235
	Metabolic 1 Subclinical Condition				0.820	0.158
	Metabolic >1 Subclinical Condition				0.520	0.105
	Metabolic NEFA > 0.70 mEq/l				0.420	0.084
	Metabolic BHBA > 10 mg/dl				0.690	0.112
	Metabolic Ca < 2.14 mmol/l				0.890	0.153
	General 1 postpartum disease				0.640	0.110
	General > 1 postpartum disease				0.340	0.089
Santos et al:		2009	8	5,719		
	Physical Dystocia				0.750	0.064
	Infectious Metritis				0.660	0.056
	Infectious Endometritis				0.620	0.056
	Infectious Fever >39.5°C				0.600	0.043
	Infectious Mastitis				0.840	0.117
	Infectious Pneumonia				0.630	0.242
	Metabolic Ketosis				0.500	0.082
	Metabolic Digestive Problem				0.780	0.224
	Physical Lameness				0.570	0.094
	General 1 postpartum disease				0.790	0.056
	General > 1 postpartum disease				0.570	0.054
Toni et al:		2015	3	1498		
	Infectious Metritis				0.640	0.071
	Infectious Endometritis				0.340	0.056
	Metabolic Cystic Ovaries				0.260	0.037
	Metabolic Anestrus				0.380	0.089
	Physical Lameness				0.540	0.041
Harman et al:		1996	6,227	44,450		
	Infectious Endometritis				0.235	0.036
	Infectious Other disease				0.653	0.128
	Infectious Other infertility disease				0.342	0.044
	Infectious Mastitis				0.786	0.058
	Metabolic Anestrus				0.418	0.026
	Metabolic Cystic Ovaries				0.439	0.021
	Metabolic Ketosis				0.786	0.041
	Physical Lameness				0.515	0.172
Francos and Mayer 1988			NR	14,573		
	Infectious Metritis				0.303	0.022

	Infectious Endometritis			0.381	0.012
	Metabolic	Retained Placenta		0.363	0.012
	Metabolic	Anestrus		0.431	0.008
Lee et al: 1989		5	1,059		
	Infectious Metritis			0.538	0.177
	Infectious Endometritis			0.709	0.103
	Metabolic	Retained Placenta		0.493	0.122
	Metabolic	Cystic Ovaries		0.538	0.137
	Physical	Lameness		0.527	0.165
Leblanc et al. 2002		27	1,865		
	Infectious Endometritis			0.700	0.083
Ferguson et al. 1995		4	566		
	Infectious Fever > 39.5°C			0.350	0.075
Ospina et al: 2010		91	2,770		
	Metabolic	NEFA > 0.27	1 week prior	0.681	0.095
	Metabolic	NEFA > 0.72	1 week after	0.730	0.119
	Metabolic	BHBA > 10 mg/dl	1 week	0.873	0.176
	BCS	BCS ≥ 3.75		1.041	0.239
Chapinal et al: 2012		55	1,919		
	BCS	BCS ≥ 4	versus <	1.00	
	BCS	BCS 3.25-3.75	vs ≥ 4.0	0.710	0.089
	BCS	BCS < 3.0	versus ≥ 4.0	0.540	0.084
	Metabolic	Ca < 2.3 mmol/l	week-1	0.670	0.105
	Metabolic	Ca < 2.2 mmol/l	week +1	0.770	0.096
Kim and Jeong 2019		2	790		
	General	One disease	postpartum	0.550	0.089
	BCS	BCS at AI <3	versus >3	0.640	0.119
	BCS	BCS at 30 day < 2.75	versus >2.75	0.792	0.155
Loeffler et al:		43	9,369		
	Infectious	Metritis		0.740	0.099
	Infectious	Endometritis		0.670	0.128
	Infectious	Mastitis		0.530	0.110
	Metabolic	Cystic Ovarian Disease		0.530	0.115
	BCS	BCS ≤ 2.5	versus 2.75-3.25	0.650	0.117
	BCS	BCS ≥ 3.5	versus 2.73-3.25	0.740	0.161
Bruinje et al:		2017	2	748	
	Metabolic	no luteal phase	versus 1 normal	0.260	0.059
	Metabolic	one abnormal luteal phase			
			versus none	0.746	0.119
	Metabolic	no luteal phase	versus 2 normal	0.381	0.066
	Metabolic	ovulation ≥ 63 days	versus earlier	0.461	0.115
Barletta et al.		2017	1	232	
	BCS	BCS loss	vs BCS gain	0.198	0.068
	BCS	BCS maintain	versus BCS gain	0.326	0.130
Santos et al:		2009	4	6396	
	Anestrus	No ovulation	65 d versus ovn	0.584	0.038
	BCS Calving	BCS < 3	versus BCS 3 - 3.5	0.848	0.060
	BCS Calving	BCS > 3.75	versus BCS 3 - 3.5	1.058	0.072
	BCS at AI	BCS < 3	versus 3 - 3.5	0.727	0.403
	BCS at AI	BCS > 3.75	versus 3 - 3.5	1.236	0.119

¹Papers reporting hazard of pregnancy or relative risk converted to odds ratio using the formula OR = (RR - RR*CR reference group)/(1 - RR*CR reference group); CR = conception rate; RR = relative risk

Cows that had dystocia, 1 postpartum disease, multiple postpartum diseases, metabolic stress, lameness, and ovulatory dysfunction have reduced CR at first insemination.

Body condition at calving, BC at insemination, and BC loss have variable effects on CR to first insemination. In general, BC < 2.5 and BC loss ≥ 1 unit have lower odds of conceiving at first insemination. Cows with BC > 3.75 at calving and that have maintained BC from calving to first insemination have CR that are better or not different from cows with no health problems postcalving.

Milk production (Figure 2) for cows with no postpartum problem compared to cows that had a health issue or delivered twin calves (897 total calving, milk production was from 841 cows distributed as follows; normal (452) metritis (131), retained placenta (106), dystocia (168), milk fever (15), ketosis (57), displaced abomasum (85), twins (47)). Milk production was reduced in cows with problems in the first 10 weeks postcalving, reducing total lactation yield in 44 weeks by 439 kg of milk.

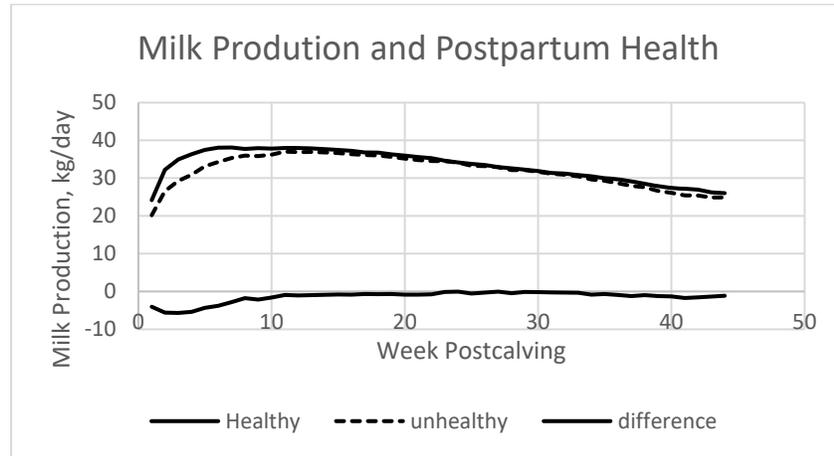


Figure 2. Milk production by week postcalving for cows with no postpartum disease and cows with a problems postcalving (metritis, retained placenta, dystocia, ketosis, displaced abomasum, and twins). Data from University of Pennsylvania Marshak Dairy, 900 cows. Total difference in milk -439 kg over 44 weeks postcalving.

A random effects model was used to calculate an overall OR of CR for cows with postpartum problems and BC change. General groupings were constructed for the data (Table 3).

Table 3. Random effects model across studies for the mean odds ratio (OR) for postpartum conditions.

Condition postpartum	OR	SE	95% Confidence Range	
anestrus, COD, nonovulatory	0.466	0.048	0.373	0.559
BC change <-1 unit from calving	0.440	0.263	-0.075	0.955
BC change ≥ -1 unit from calving	0.502	0.089	0.327	0.677
BC at AI 2.75 - 3.25	0.581	0.665	-0.722	1.884
BC at calving 2.75 - 3.5	0.648	0.197	0.263	1.033
BC at calving ≥ 3.75	0.936	0.083	0.774	1.098
BC at calving < 3	0.616	0.117	0.387	0.846
BC at AI ≥ 3.75	0.939	0.185	0.576	1.301
BC at AI < 3	0.671	0.129	0.418	0.924
One postpartum disease	0.617	0.104	0.412	0.821
More than one postpartum disease	0.390	0.113	0.169	0.612
Metritis, Mastitis postpartum	0.478	0.043	0.394	0.561
Postpartum ketosis, elevated NEFA	0.499	0.044	0.413	0.585
Two metabolic conditions	0.502	0.216	0.080	0.925
Dystocia, lameness, still birth	0.610	0.063	0.487	0.732
Overall Mean	0.542	0.020	0.502	0.581

Cod = cystic ovarian disorder

Nonovulatory = no ovulation prior to first insemination

BC = body condition, scale 1 to 5, with 1 emaciated, 2 thin, 3 average, 4 fat, and 5 obese

AI = artificial insemination

Overall, having a postpartum problem, significant BC loss, or having thin BC at insemination had an OR of 0.542 (SE 0.020) on insemination to first insemination. If CR in normal cows is 50%, the CR in

problem cows would be 35.1%. If heat detection (insemination rates) were similar in both groups of cows, which is unlikely as cows with reproductive problems often have delayed first ovulation, normal cows would achieve a PR of 25.0% if CR was 33.3% and insemination rates 75%. Cows with reproductive problems would have a CR of 21.3%, which would result in a PR of 16.0% if insemination rates were 75%. This would correspond to a hazard rate of pregnancy of 0.76 compared to normal cows. Effects on survival curves for normal cows and reproductive problem cows are shown (Figure 3). The lower PR reduces milk produced per day due to longer calving intervals, fewer calves born per year, and a higher risk of culling due to more open cows at or beyond 230 days postcalving. In addition, cows with reproductive problems due to health issues produce about 439 kg less milk over a 305 days lactation, primarily due to lower yields in the first 10 weeks postcalving. The result would be a reduction in income of \$404.00 per cow per year (milk income, \$0.17/lb; calf value, \$150/head; replacement costs, \$750/cull cow).

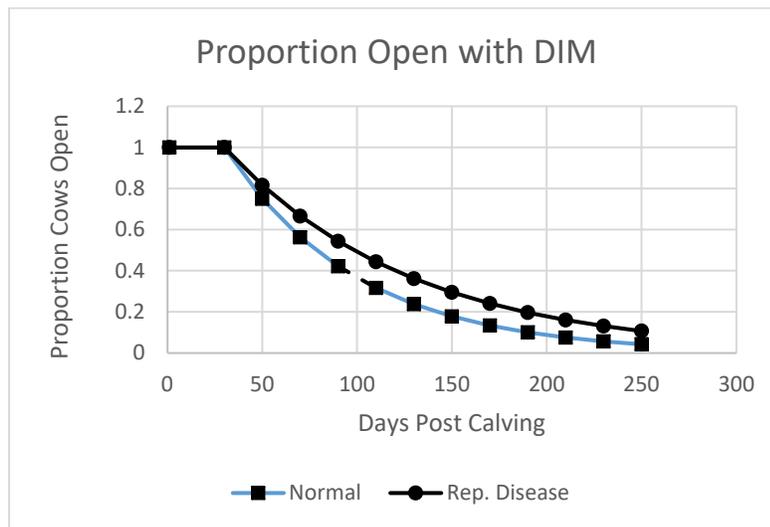


Figure 3. Failure time (pregnancy rate) curves for cows with normal postpartum conditions (■) versus cows with reproductive disease or late first ovulation (●). Normal cows have a pregnancy rate of 25.0% (Insemination rates of 75% and CR of 33.3%), whereas the reproductive disease cows have a pregnancy rate of 18.4% (insemination rate of 75% and CR of 24.5%, based on a mean OR of 0.65 compared to normal cows for reproductive disease). Voluntary wait period is 50 days. The hazard rate of pregnancy for the reproductive disease cows is 0.828 relative to the normal cows.

Initiation of ovulation

Reinitiation of ovarian cycling early postpartum is critical for full restoration of fertility.^{6,10-14,48} Ovulation of a healthy ovum capable of fertilization and normal embryonic development is essential for maintenance of pregnancy.⁴⁷ Follicular development affects the quality of the ovum and of the corpus luteum that develops following ovulation, influencing embryonic development, progesterone secretion, and the maintenance of pregnancy.⁴⁷ Typically, it has been observed that several estrous cycles prior to the breeding period are necessary to ensure a healthy ovum and sufficient progesterone secretion to support pregnancy.⁶ To that end, earlier first ovulation and normal luteal phases prior to first insemination are associated with higher pregnancy rates.⁴⁹ The degree of energy balance nadir,¹⁶ the days to energy balance nadir,¹³ and uterine bacterial contamination can influence to day to first ovulation⁵⁰ and the quality of ovum and progesterone produced by the subsequent corpus luteum.⁸

Negative energy balance, the difference in energy output in milk minus energy intake from feed, is a factor influencing resumption of luteal activity postpartum and subsequent fertility.¹³ Majority of cows experience negative energy balance postcalving, with the nadir typically occurring within the second week (Figure 4). Majority of cows return to positive energy balance by week 8, with a range from 4 - 12 weeks (Figure 4). Cows tolerate moderate degrees of negative energy balance in the early

postpartum period, but there seems to be a threshold that impairs ovarian function and fertility.¹⁶ Days to first ovulation were negatively correlated with the nadir of negative energy balance, rather than the time.¹⁶ The greater the negative energy balance, the longer the days to first ovulation. The greater the degree of negative energy balance postpartum, the greater the serum concentrations of NEFA and BHBA, and the lower the concentration of glucose, insulin, and IGF-1.^{10-12,15,19} Negative energy balance is greater in cows with greater BC loss over 30 days postcalving. Cows with NEFA > 0.7 mEq/L, BHBA >10 mg/dl had lower serum concentrations of insulin, IGF-1, and glucose in the first 1 - 3 weeks postcalving.

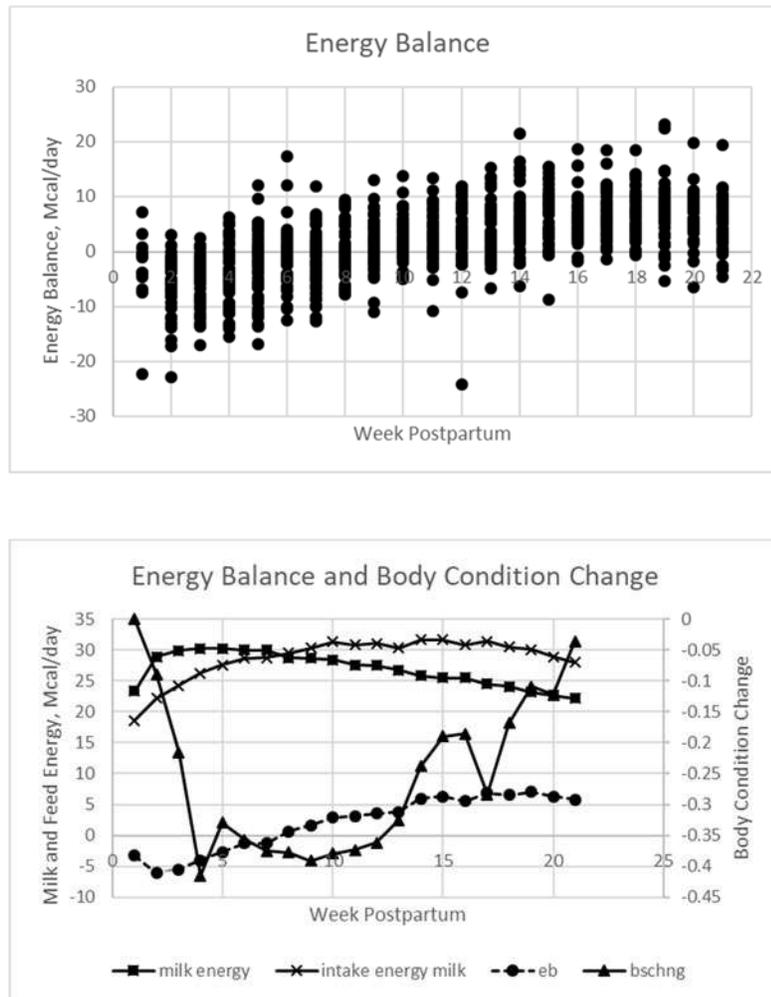


Figure 4. Daily energy balance (energy intake in feed - energy output in milk) by week postpartum in 40 lactating Holstein dairy cows (upper panel). Energy output in milk and energy intake from feed (Mcal/day) to energy balance (eb) and body condition change from calving body condition score (bschng) on a 5-point scale, using quarter-point increments, by week postcalving (lower panel).

It is not possible to measure energy balance on a farm, but body condition score (BCS) is a good estimator of body fat,⁵¹ and change in BCS is a good estimate of fat mobilization and cumulative negative energy balance.^{52,53} One unit of BC loss was associated with 42 or 54 kg of fat mobilized to support milk production, corresponding to 420 and 564 Mcal of net energy of lactation used for milk production from body fat.^{52,53} These estimates of tissue loss and energy depend upon how fat the cow is; a cow with a BCS of 4 will lose more fat with a higher energy value when losing 1 unit of condition than a cow with a BCS

of 3.⁵⁴ In addition, cows fat at calving will lose more BC than thinner cows. Generally, 1 unit of BC loss corresponds to -300 to -500 Mcal of cumulative negative energy balance for cows with a BCS of 2.5 - 4.0.

Body condition loss is greatest between calving and 30 days in milk, then tends to flatten over the next 4 - 8 weeks before increasing, as cows accumulate positive energy balance (Figure 4). Cows seem to tolerate up to ¾ of BCS loss before a reduction in first service conception rate is observed. When BC loss is > ¾ of BCS, the odds ratio of first service conception rate (FSTCR) is 0.34 - 0.50 relative to no change in BC in the first month postcalving. Meta-analysis (15 studies) of BC and BC loss observed heterogeneity of BC loss and FSTCR, with significant fertility reduction in cows with low BC at calving or at first AI (< 2.5) and in cows with BC loss > 1 unit of BCS.⁴⁴ This would tend to support the observation that cows tolerate ¾ of BC loss. Thinner cows, particularly < 2.5 BC, had lower fertility than cows with greater BC.^{44,45} Cows in thinner (≤ 3.0) or moderate BCS (3.35 - 3.75) at calving had a lower odds of pregnancy at first service (OR = 0.54, OR 0.71, respectively) compared to fat cows (≥ 4.0 BCS) at calving.²³ Body condition loss and BC at first insemination create the heterogeneity in associating condition score with conception rates.

The influence of energy balance on FSTCR may operate through delays in first ovulation. Cows that ovulate after 35 days postpartum have lower first service conception and lower pregnancy rates.⁴⁸ Longer days to first ovulation is associated with lower energy balance, later days to energy balance nadir, lower body condition score at 30 days postpartum, metritis, abnormal calving, ketosis, and increased serum NEFA in the first week postpartum.^{10-14,16,40,41,46,49,50,56} Longer days to first ovulation postcalving is consistently associated with lower FSTCR.

Ovulation of the first dominant follicle occurred at 21 days postcalving in 30 - 50% of cows^{10-14,16,40,41,46,49,50,56} (Figure 5). At 50 days postcalving, 20% of cows had not ovulated (Figure 5) resulting in lower first service conception rates. Cows that were in synchronized ovulation and timed artificial insemination program had a FSTCR of ~ 20% lower than cycling contemporary cows. Metritis,

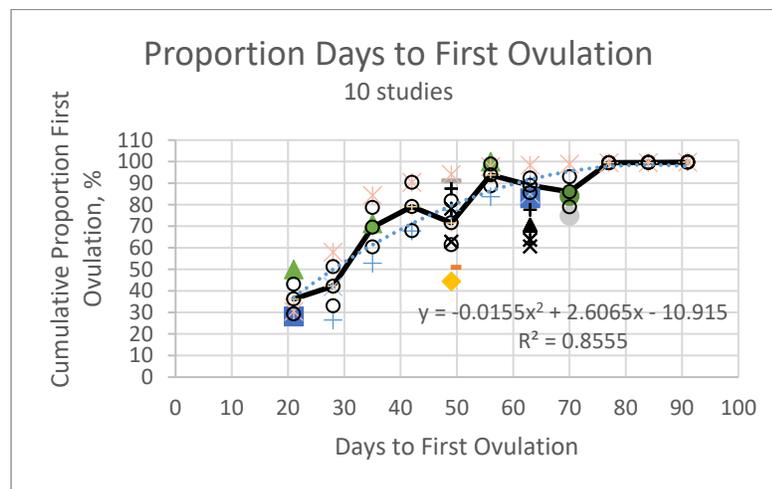


Figure 5. Cumulative proportion of cows with first ovulation by days postcalving across studies.^{10-14,16,40,41,46,49,50,56}

ketosis, digestive upsets, and calving difficulties increased the proportion of anovulatory cows at 50 days postcalving to 30% and further reduction in FSTCR occurred in cows that had greater risk of endometritis.

Embryonic mortality

Postpartum conditions reduced conception at first insemination with higher pregnancy loss after insemination.^{5,47,57} Losses in the first week were due to fertilization failure, from days 8 - 27 due to failure of maternal recognition of pregnancy, from 28 - 60 days due to improper placental development, and from 60 - 90 days, due to placental growth.⁴⁷ On dairy farms, fertilization failure and

embryo losses are not apparent, except as return to estrus within 24 days from insemination. Losses from 8 - 27 days may only be apparent as returns to estrus between 25 - 35 days after insemination, but inseminations in this time period may be confounded with prior inseminations of cows that were not truly in estrus. Losses from 28 - 60 days and 60 - 90 days would be observable as cows checked pregnant at an examination from 28 - 42 days postinsemination then either returning to estrus after examination or not pregnant at a subsequent examination. These losses were characterized as late embryonic losses based on the classification; early embryonic loss (15 - 17 days postinsemination), late embryonic loss (17 - 42 days postinsemination), and fetal loss (after 50 days postinsemination).⁵⁷ Due to pregnancy losses and fertilization failure following insemination, it is estimated that only about 30 - 35% of inseminations result in a live calf.^{5,47} With this degree of loss, it would require 5 or more inseminations to achieve 85% probability of inseminated cows recalving.

On most farms, with routine veterinary reproductive programs, pregnancy loss will be most apparent from 30 - 60 days after pregnancy examination. Typically, cows are first examined for pregnancy ~ 28 - 42 days postinsemination. Pregnancy loss was ~ 12% at 60 days postinsemination after a pregnancy examination at 30 days postinsemination.⁴⁷ However, this may be influenced by heat stress and prior postpartum conditions. Postpartum conditions will not only lower conception rates, they will also increase late embryonic loss.

Conditions (Figure 6) that result in pregnancy loss were classified.^{45,49,59-84} Classifications were organized into two groupings: "no major problem:" (bcsmain = maintenance of body condition from calving; norm = cows with no health problems postcalving and ovulatory before insemination; bcsgain = cows gaining body condition from calving to insemination; bcslloss = cows with body condition loss from calving with no other identified health problems; tai = ovulatory cows inseminated on timed insemination; ov = cows that had ovulated prior to first insemination); "problem:" (dis = cows with 1 postpartum disease; met = cows with subclinical metabolic disease; phy = cows with a physical problem such as lameness or dystocia; inf = cows with metritis, endometritis, mastitis, pneumonia; multdis = cows with more than one condition post calving; abn = general classification for other abnormal conditions postcalving). Classes were examined using a random effects model in SAS model with study as random effect. A total of 94 observations were analyzed from 28 studies.

Data from cows classified as "no major problem" had a pregnancy loss of 16.5% (SEM \pm 2.3%) similar to 12% reported.⁴⁷ However, cows with problems postcalving had a loss of 27.2% (SEM \pm 3.1%). Cows that had not ovulated by the voluntary wait period had a pregnancy loss of 20.3%, intermediate to cows without a problem and cows with problems postcalving. This analysis should be viewed cautiously, as more descriptive analysis is needed. However, it points that the loss of pregnancy in cows with no problems are significant and losses in cows with problems are about double the loss in cows with no problems.

Proportion of pregnancy loss 30 days from first insemination (Figure 7) suggested wide variation, with higher losses in cows that had a lower CR at 30 days postinsemination.

Overall impact on herd performance

Most dairy farms calve cows year-round, have cows of multiple parities, and have a proportion of cows that experienced health problems postcalving. In addition, cows with no apparent health issues may

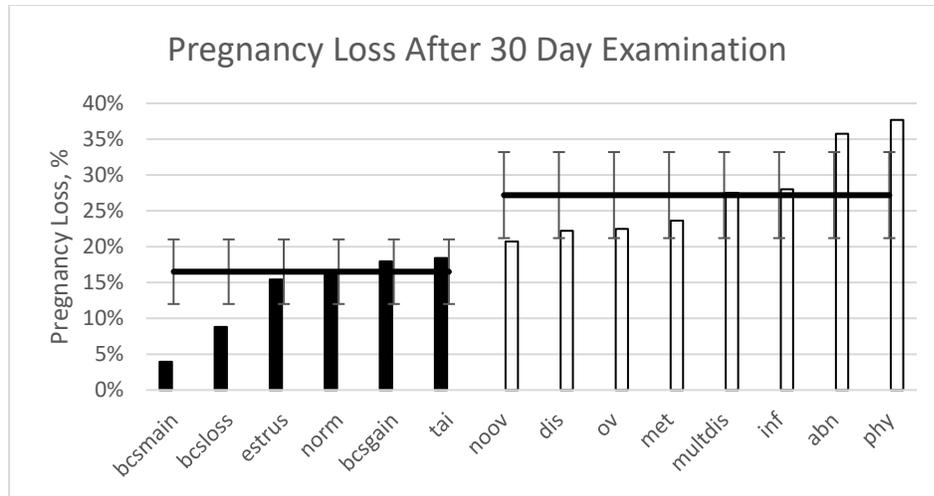


Figure 6. Random effects model of the proportion of pregnancies lost from first insemination after a "30" day initial pregnancy examination for categories of conditions: bcsmain = maintenance of body condition from calving; norm=cows with no health problems postcalving and ovulatory before insemination; bcsgain= cows gaining body condition from calving to insemination; bcsgain = cows with body condition loss from calving with no other identified health problems; tai = ovulatory cows inseminated on timed insemination; ov = cows that had ovulated prior to first insemination; dis = cows with one postpartum disease; met = cows with subclinical metabolic disease; phy = cows with a physical problem such as lameness or dystocia; inf = cows with metritis, endometritis, mastitis, pneumonia; multidis = cows with more than one condition postcalving; abn = general classification for other abnormal conditions postcalving. The lines represent a mean loss of 16.5% (SE 2.3%) and 27.2% (SE 3.1%), with error bars representing the 95% confidence range for the two estimates.

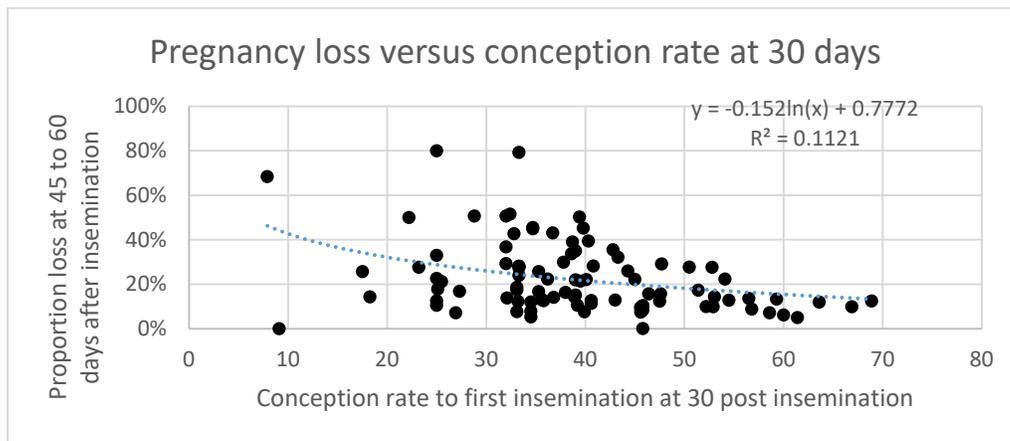


Figure 7. Proportion of pregnancies lost against the conception rate at 30 days after insemination.

not ovulate by 50 days postcalving. Healthy cows that ovulates prior to 50 days postcalving had the best fertility in the herd and the lowest pregnancy loss. Given the pattern of first ovulation (Figure 5), 81% of healthy cows would ovulate prior to 50 days postpartum and 19% of cows would not. For cows with health problems, 67% would ovulate by 50 days and 33% would not. Cows with delayed ovulation and health issues will have an OR of 0.54 for first service CR. Cows with more than one health problem or a health problem combined with delayed ovulation will have an OR of FSTCR of 0.29 (Table 3). The impact on FSTCR would be as follows: if healthy cows have a FSTCR of 50%, then healthy cows with ovulation later than 50 days would have a FSTCR of 35%, as would cows with one health problem that ovulated prior to 50 days. Cows with multiple health problems and with late first ovulation would have a FSTCR of 22%. Pregnancy losses will also be influenced by these conditions. Cows with no health problems that ovulated prior to 50 days would have a pregnancy loss of 16.5%, which would reduce

Table 4. Impact of heat detection rate (insemination rate), conception rate based on a pregnancy exam at 30 days postinsemination, OR of CR of 0.54 in cows with delayed ovulation after 50 days postpartum and with one health problem, and an OR of 0.28 in cows with two or more health problems or health problem and delayed ovulation. Pregnancy loss after 30 days was 16.5% for cows with no health problems and 27.2% for cows with delayed ovulation or one or more health problems. Break even dollars were calculated relative to a PR of 25%.

HDR %	Healthy Cows % of cows calving	CR Needed to break even % of insemination pregnant at 30 days
50	50	76
50	60	73
50	70	69
50	80	66
60	50	70
60	60	67
60	70	63
60	80	60
70	50	64
70	60	61
70	70	57
70	80	54
80	50	58
80	60	55
80	70	51
80	80	48

pregnancy following first insemination from 50 to 42%. Healthy cows that ovulated after 50 days appear to have a pregnancy loss by 60 days of 20%, resulting in a pregnancy outcome at 60 days of 28%. Cows with health problems have a pregnancy loss of 28%; pregnancy at 60 days would be 25%. Pregnancy at 60 days in cows with multiple problems would be 20%.

Herd reproductive performance would be a composite of the proportion of healthy cows and cows with significant body condition loss and severe negative energy balance, infectious disease (metritis, endometritis, mastitis), metabolic disease (ketosis, displaced abomasum, lameness), subclinical metabolic conditions (elevated NEFA and BHBA, and hypocalcemia). The standard of fertility in the herd would be healthy cows with first ovulation prior to 50 days postcalving. All other groups of cows would have lower reproductive performance, both in terms of pregnancy at 30 days postinsemination and maintenance of pregnancy to 60 days postinsemination. Depending on the FSTCR in healthy cows and the proportion of healthy cows, the ability to achieve an optimal economic return on reproduction will be limited.

Impact of heat detection rate (HDR, insemination rate) on CR in healthy cows at 30 days postinsemination to achieve a PR of 25% is summarized (Table 4). It requires a herd with more than 50% of cows calving with no health problem and an insemination rate of 70% or better to have a CR in healthy cows below 55% to achieve a herd PR of 25%. This is a challenge on many dairy farms.

Typically, on most farms, only 50% of cows have no problems postcalving. Average HDR are below 50% according to DHIA summary statistics. Therefore, reproductive performance is below a pregnancy rate of 25% on most farms. This represents a significant loss of income. There are major opportunities to improve reproduction by improving health of cows in transition from the dry period to lactation and employing programs to ensure high rates of insemination.

Conclusion

To prevent health problems postcalving in less than 50% of cows calving requires a sound management program prior to calving and in the first month postcalving. Housing, feed bunk access, and adequately formulated rations are a must for decreasing health conditions postcalving. It is beyond the scope of this paper to identify adequate rations for late pregnancy, but those are described elsewhere.⁸⁵⁻⁸⁷

Conflict of interest

There are no conflicts of interest to declare.

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Reproductive management in dairy herds

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Abstract

Reproduction influences farm profitability. Cows should become pregnant between 55 and 115 days postcalving to optimize milk production, calves born per year and minimize culls for reproductive failure. Pregnancy rate, heat (estrus) detection rate times conception, is the critical driver of reproductive efficiency. Economic returns associated with reproduction are optimal when pregnancy rate is > 25%. To achieve a pregnancy rate > 25%, conception rate needs to be > 33% and first insemination heat detection needs to be > 70%. Herd managers should select a voluntary wait period between 50 - 70 days that optimizes conception rate and use a management program to control first insemination intensity. Pregnancy examination should be scheduled to control days between inseminations.

Keywords: Cattle, reproductive management, pregnancy rate

Introduction

Milk sales account for 80 - 90% of income on dairy farms and sale of calves and cull cows account for 10 - 20% of income.¹ Milk sales are a function of the number of cows milking, parity, average days in milk, management, and genetic merit. However, milk production is dependent on the successful birth of a calf, that makes reproduction an essential component of a successful dairy operation. Due to the shape of the lactation curve, peak production is reached by 30 - 50 days postcalving and then declines at a consistent rate; therefore, cows produce milk most efficiently when they calve every 11 - 13 months. Since pregnancy gestation averages ~ 280 days, the goal of reproductive programs on dairy farms should be for pregnancy to occur between 55 - 115 days postcalving. Since uterine involution and resumption of ovarian cycling are not complete until 30 - 50 days postcalving, management programs should begin insemination 50 - 70 days postcalving to achieve optimal intervals between successive calving.

To become pregnant, cows must be inseminated in estrus, that occurs every 21 days in a cow (range; 18 - 24 day).² Standing estrus is relatively short, lasting 5.1 - 10.6 hours.³ Depending on the number of cows around estrus, the number of mounts per estrus period may range from 6.2 - 12.8 mounts per cow.⁴ A mount may last for < 20 seconds, so observation to detect a cow in estrus needs to be committed for at least 15 minutes at a time and occur 3 - 4 times in a day.

Various factors influence the intensity of expressed estrus by a cow such as number of cows around estrus, flooring, and level of milk production.^{2,3} Having more than 2 cows in estrus increases activity and the probability of observing estrus. Cows with greater milk production have lower serum estrogen, shorter periods of estrus expression, and fewer estrus mounts.^{4,5,6}

A major challenge in reproductive management is observing cows in estrus. Average heat (estrus) detection rate for 9,480 herds in the Dairy Records Management Service (DRMS, Raleigh, NC) in the NE is 45.1%. Multiple factors influence efficiency of estrus detection. Housing, flooring, cattle grouping, number of cows in and around estrus, level of milk production, parity, and frequency and intensity of observation all contribute to the probability of observing cows in estrus.⁶ However, the major factor in low estrus detection is failure to commit sufficient time throughout the day to detect cows in estrus. Particularly as herds have increased in size and cows are spread across multiple groups, committing labor to just watch for estrus is a low priority on many farms. Many approaches may be employed to improve estrus detection on dairy farms. There is visual observation, heat detection aids, radio telemetry, activity monitors, progesterone biosensors, and estrus synchronization programs.⁸⁻¹¹

Evaluating the efficiency of estrus detection is important. Estrus detection needs to be quantitated to evaluate the efficiency of a reproductive program. Days open and calving interval are outcomes of management and are not critical control points for reproductive control. Heat detection is the dominant management control point of reproduction and it must be quantitated to assess a program on a dairy farm. Various methods have been used to estimate heat detection rates (HDR) from herd records.¹² A simple

method is to distribute first inseminations from the VWP by 21-day periods and calculate the number of animals inseminated within each 21-day period by the number of available animals to inseminate. This will give a value for first insemination heat detection rate (FSTHDR). Distributing days between sequential inseminations by periods < 10, 10 - 17, 18 - 24, 25 - 35, 36 - 48 and > 48 days gives an estimate of estrus detection for repeat inseminations (18 - 24 and 36 - 48 days), heat detection errors (< 10 and 10 - 17 days), early pregnancy losses (25 - 35 days), and gross inefficiency in repeat insemination efficiency (RPTHDR) (> 48 days).

Management has direct control of detecting cows in estrus for insemination. Since pregnancy depends on insemination, cows have to be observed in estrus to initiate reproductive outcomes. Conception, establishing a pregnancy, is the second critical component of a herd program. Conception rate (CR) to first service for 9,480 herds in the DRMS in the NE is 42%. With an HDR of 45% and a CR of 42%, pregnancy rate would be 18.9%. The question is how well does this approach a reproductive optimum?

Ultimately, herd reproductive performance is a function of heat detection rate (HDR) and CR. Since estrus is a periodic event, occurring every 21 days, the combination of HDR and CR define the pregnancy rate (PR), that is the proportion of open cows that become pregnant every 21 days.^{13,14} The reported PR from the 9,480 herds in the DRMS records is 19.4%. This is slightly higher than the prior estimate of 18.9%, as culling cows with reproductive problems would result in a slightly higher PR than HDR x CR.

Pregnancy rate determines the economic value of reproduction.^{1,14,15} The proportion of cows pregnant every 21 days following the voluntary wait period (VWP) determines the average milk produced per day (due to the shape of the lactation curve and period of recalving), the number of calves born per year, and the number of animals culled for reproductive failure. These contribute to the value of getting a cow pregnant within each 21-day period from the VWP. The proportion of open cows pregnant with each 21-day window sums to determine the overall herd value of reproduction.

Figures 1a and 1b display income over feed cost for PR from 7.5 - 100% for five milk production classes (305 day production): 21,000, 23,000, 26,000, 28,500, and 33,000 lb. Milk price was \$20/CWT, calf value was \$175/head; heifer rearing cost was \$1500/head and cull cow value was \$750/head. Lactating cow feed cost was \$0.12/lb of dry matter and dry cow feed cost was \$0.06/lb of dry matter. Income is higher with greater milk production and pregnancy rate (Figure 1a). Marginal value decreases more steeply when PR is < 25%, whereas the change in value above 25% is less (Figure 1c). Therefore, economically, the goal for herd management is to achieve a 25% PR or greater. Furthermore, with a PR of 19.4%, the average DRMS herd is losing \$165 a year due to low reproductive efficiency.

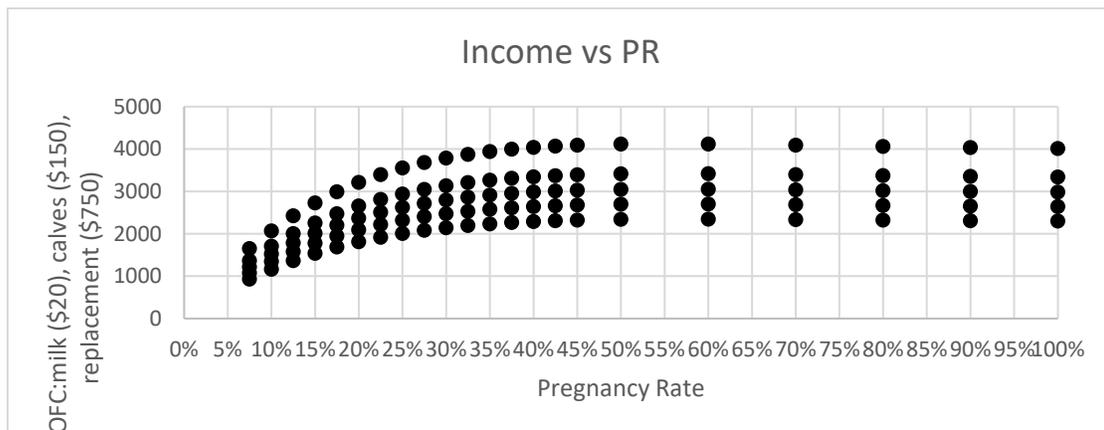


Figure 1a. Income over feed cost for five levels of milk production for pregnancy rates varying from 7.5 to 100%. Milk price is \$20/CWT, calf value is \$175/head; heifer rear cost is \$1500/head and cull cow value is \$750/head. Lactating feed cost is \$0.12/lb of dry matter and dry cow feed cost is \$0.06/lb of dry matter. Milk production M305 is 21,000, 23,000, 26,000, 28,500, or 33,000 lb per 305 days of production.

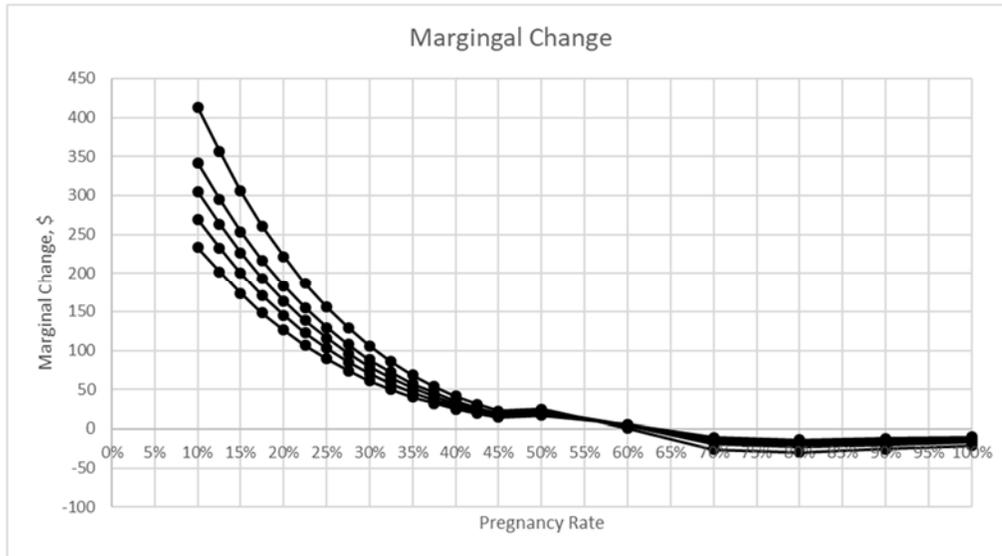


Figure 1b. Marginal change in income over feed cost as a function of pregnancy rate (same assumptions as Figure 1a).

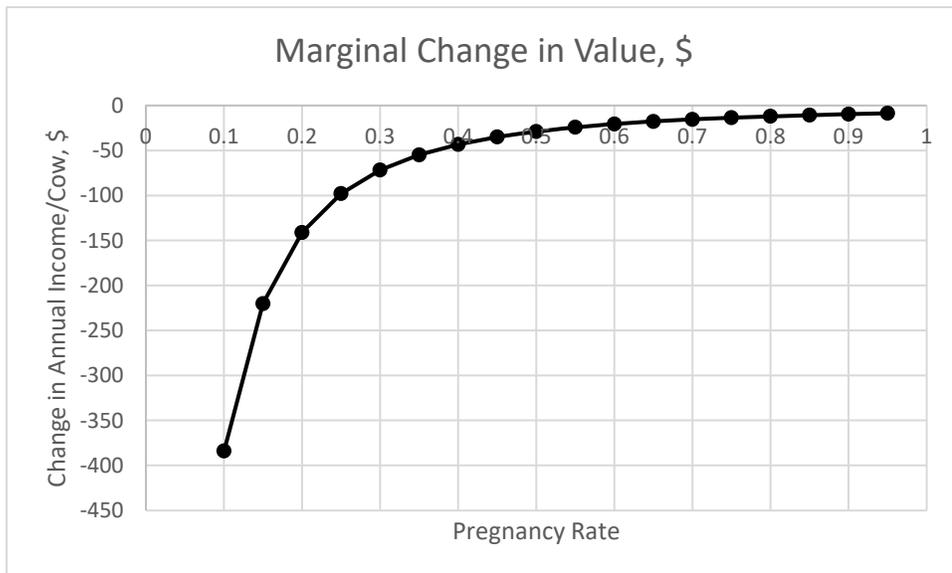


Figure 1c. Marginal change in value as pregnancy rate declines from 100%. Losses below a pregnancy rate of 25% are six-fold the losses above a pregnancy rate of 25%.

Factors influencing pregnancy

Pregnancy rate is a function of HDR and CR.^{13,14} Heat detection rate has different attributes, depending whether detecting estrus in cows for first insemination versus detecting cows in estrus postinsemination. Cows for first insemination are not pregnant and therefore may be induced into estrus using prostaglandin or combinations of prostaglandin (PGF_{2α}) and gonadotrophin releasing hormone (GnRH). Therefore, first inseminations may be clustered in groups of cows within a 21-day window from the VWP. However, once inseminated, a cow cannot be induced into estrus until the farm manager is certain the cow is not pregnant. Return to estrus at 21 days postinsemination must be based on visual observation or an analysis of hormonal indicators of nonpregnancy status, such as declines in progesterone or bovine pregnancy glycoproteins. Most pregnancy tests are not accurate until 28 - 35 days postinsemination. Therefore, clustering of open cows for reinsemination cannot be done until they are confirmed not pregnant. First insemination HDR (FSTHDR) can be clustered in a 21-day window from

the VWP; repeat HDR (RPTHDR) for reinsemination cannot be clustered in a 21-day period, but at best at a 42 - 48 day period. In addition 100% of cows are available for first insemination whereas only $(1 - \text{FSTCR}) * 100\%$ of cows are available for second insemination, with a declining proportion at each sequential service.

Figures 2a, b, c present pregnancy rate as a function of conception rate across all services (2a), of first insemination heat detection rate (2b), and of repeat heat detection rate (2c) for 534 observations from 325 herds, largely in Pennsylvania. To achieve a PR of 25% or greater, CR needs to be $> 33 - 35\%$. Conception may be greater than this and not achieve a 25% PR, due to low rates of heat detection. To achieve a PR of 25%, FSTHDR needs to be $> 70\%$ (Figure 2b) and a higher proportion of herds achieve a PR of 25% when FSTHDR is 100%; that occurs when a synchronized first insemination program is employed. To achieve a PR of 25%, RPTHDR needs to be $> 50\%$ (Figure 2c); however, RPTHDR has a lower association with PR than FSTHDR and CR.

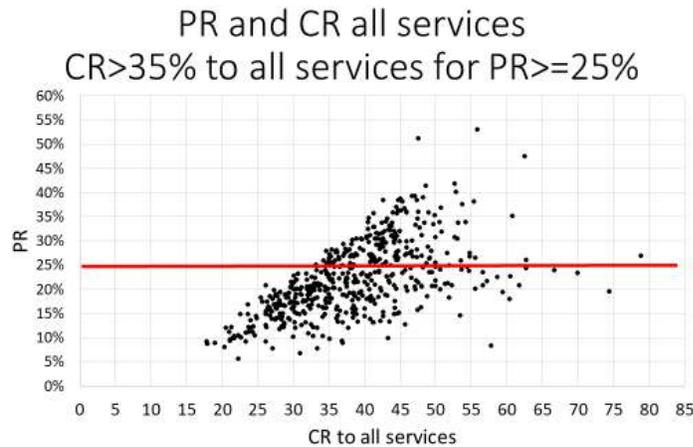


Figure 2a. Pregnancy rate for conception rate across all services for 539 herds. For a pregnancy rate of 25%, conception rate needs to be at least 33% or more. However, conception rates greater than 35% may not achieve 25% pregnancy rates due to low heat detection rates.

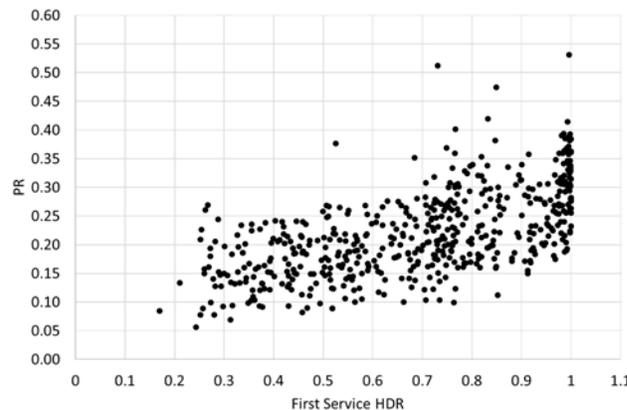


Figure 2b. Pregnancy rate for first service heat detection for 539 herds. First insemination heat detection rate has to be above 70% to achieve a 25% pregnancy rate.

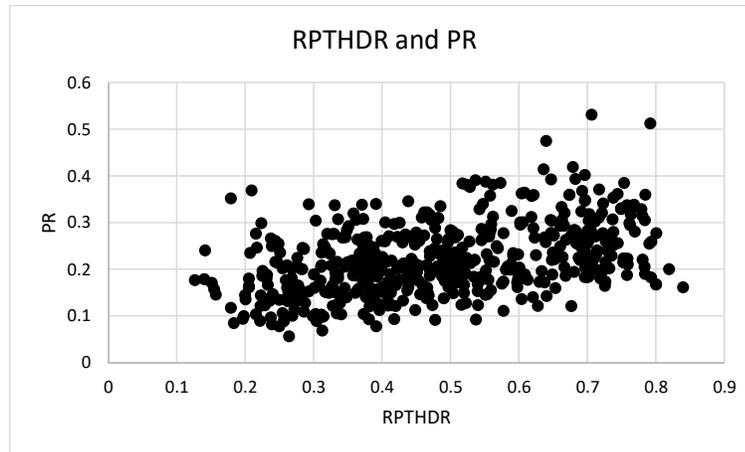


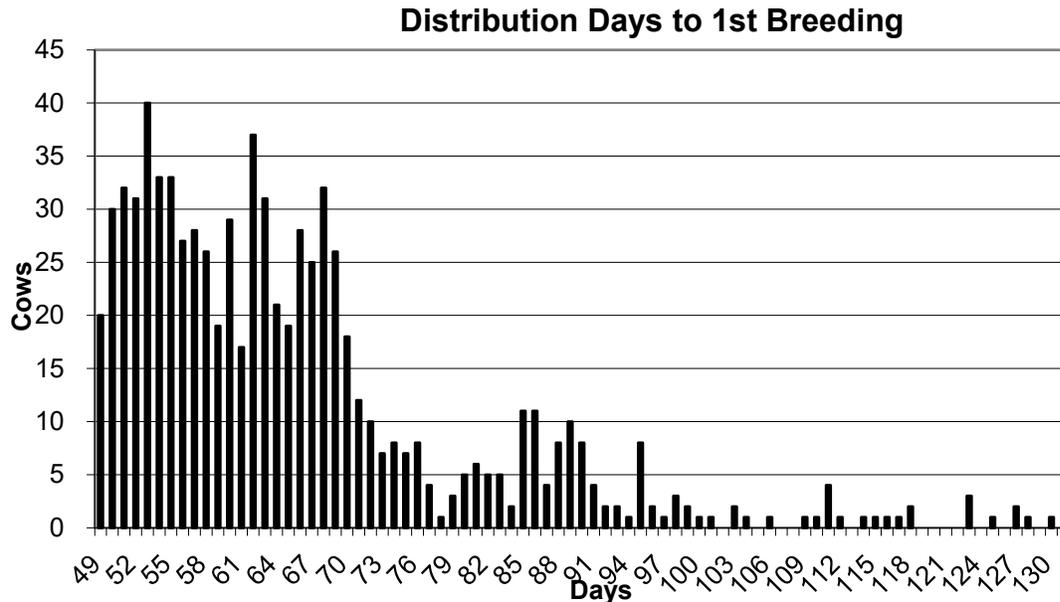
Figure 2c. Pregnancy rate for repeat heat detection rate for services after first service for 539 herds. Repeat heat detection rate needs to be greater than 50% to have a greater probability of achieving a pregnancy rate of 25%.

Mean values for herd observations with $PR \geq 25\%$ and for herd observations with $PR < 25\%$ are shown (Table 1). Except for mean days to first insemination, the proportion of interbreeding intervals less than 10 days and between 25 to 35 days and the percent of annual culls, all reproductive indices are superior for herds with $PR \geq 25\%$. Herds with a pregnancy rate of $\geq 25\%$ realized \$333/cow more income if milk price was \$20/CWT, calves had a value of \$150/head, replacement cost minus cull cow value was \$750/cow, lactation feed cost was \$0.12/lb of dry matter and dry cow feed cost was \$0.08/lb of dry matter. Although the lower pregnancy rate group produced more total milk, it occurred over a longer period of time, reducing the efficiency of milk produced per day, reducing calves born annually, and increasing replacement costs due to fewer pregnancies over a 126-day breeding period.

Table 1. Mean values for 325 herds and 534 observations for mean reproductive indices for herds with pregnancy rates equal to and $> 25\%$ and herds with $< 25\%$ pregnancy rate

Item	PR < 0.25 Mean \pm SEM		PR \geq 0.25 Mean \pm SEM		P value
Number observations	359		175		<.0001
Voluntary Wait Period, d	52.38	0.68	61.53	0.82	<.0001
Days to first insemination, d	78.97	0.70	77.56	0.83	0.0718
Proportion First Insemination Rate	0.638	0.011	0.825	0.014	<.0001
Proportion Conception Rate All Services	0.351	0.005	0.436	0.005	<.0001
Proportion Repeat Insemination Rate	0.479	0.009	0.560	0.011	<.0001
Pregnancy Rate	0.185	0.003	0.305	0.004	<.0001
Interval Between Inseminations	43.76	0.56	37.86	0.63	<.0001
Proportion of Inseminations < 10 days	0.026	0.003	0.034	0.003	0.0152
Proportion of Inseminations 10 - 17 days	0.038	0.002	0.036	0.002	0.4491
Proportion of inseminations 18 - 24 days	0.237	0.007	0.256	0.008	0.04
Proportion of inseminations 25 - 35 days	0.128	0.004	0.120	0.005	0.1875
Proportion of inseminations 36 - 48 days	0.280	0.012	0.353	0.015	<.0001
Proportion of inseminations \geq 49 days	0.294	0.009	0.218	0.011	<.0001
Pregnant after 126 days breeding period	0.693	0.004	0.880	0.005	<.0001
Calving interval, days	457.0	1.4	415.2	1.5	<.0001
Mean lactation age of herd, years	2.37	0.02	2.48	0.020	<.0001
Proportion of active cows culled	0.229	0.010	0.243	0.012	0.2952
Total milk produced/cow, kg/cow	12,490	14.9	12,044	15.0	<.0001
Milk produced per day, kg/cow	27.40	0.05	29.01	0.06	<.0001
Annual value of income /cow, \$	1,833.0	9.2	2,166.0	10.5	<.0001

Good reproductive performance is possible using visual heat detection. Figure 3 presents data from a farm for first insemination and repeat insemination using visual observation to manage insemination. Heat detection rates are >70% and PR is >25% on this farm. Good reproductive management may be achieved with visual observation. It will be reflected in first insemination and repeat insemination frequencies. First insemination frequencies will be spread somewhat uniformly over 21 day periods. Frequency distribution of first insemination by days in milk from the voluntary wait period of 48 days is shown in Figure 3 with corresponding Tables.



Distribution by 21-day periods of first inseminations from the voluntary wait period:

Heat Detection	Cows bred:		838		Total cows:		1101	
All Lactations								
Wait Period	1-48	49-69	70-90	91-111	112-132	≥ 133	Total	
Number of cows:	41	584	153	37	16	7	838	
Cumulative %:	4.9%	74.6%	92.8%	97.3%	99.2%	100.0%		
Heat detection:	4.9%	73.3%	71.8%	61.7%	69.6%	100.0%		
Mean HDE:	72.4%	95% CI:	3.1%	Range:	69.3%	<---->	75.5%	

Distribution of days between inseminations by category intervals:

Interval Analysis: Distribution of Days between Breeding							
Services Sum of 1 - 2, 2 - 3 and 3 - 4							
All Lactations							
Interval	< 10	10 - 17	18 - 24	25 - 35	36 - 48	≥ 49	N
# Cows:	37	55	476	75	102	72	
% Cows:	4.5%	6.7%	58.3%	9.2%	12.5%	8.8%	817
Long:	8.8%	Abnormal:	17.4%	Ratio:	4.7	PostHDR:	70.8

Figure 3. The graph is the frequency distribution of first insemination by days in milk from the voluntary wait period of 48 days. Grouping by 21-day periods from the VWP allows calculation of first insemination rates. The first insemination intensity (first insemination heat detection rate, FSTHDR) was 72.4%. Pregnancy rate in this herd was 31.3%. Conception rate to all services was 44.3%. Next is the frequency distribution of days between inseminations grouped by <10, 10 - 17, 18 - 24, 25 - 35 and > 48 days.

Figures 4a, b, c presents the same graphics for pregnancy rate but from a group of herds with superior reproductive performance (149 herds). A subset of these herds was reported.¹⁵ Conception rate is strongly associated with PR, and almost all these herds have a CR > 35%. Pregnancy rate is also strongly associated with FSTHDR. Repeat heat detection rate (RPTHDR) is not strongly associated with PR, but all these herds have RPTHDR greater than 50%. These herds averaged a PR of 36.0% (SD 6.0%), CR of 48.0% (SD 7.5%), FSTHDR of 91.4% (SD 9.8%), and a RPTHDR of 64.8% (SD 9.3%). These herds were large (mean size, 1530 cows) but ranged in size from 81 to 21,000 cows. Herd size does not inhibit good reproductive performance. Conception rate in these superior herds is not significantly better than CR in DRMS records. What makes these herds superior in reproductive performance is high rates of first insemination efficiency

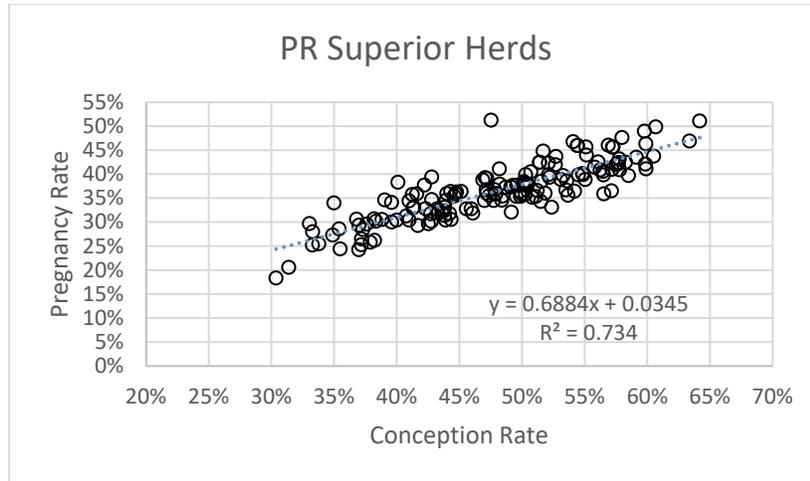


Figure 4a. Pregnancy rate and conception rate to all services for 149 herds with excellent reproductive performance from across the US. If conception rate is greater than 35%, no herd has a PR less than 25%.

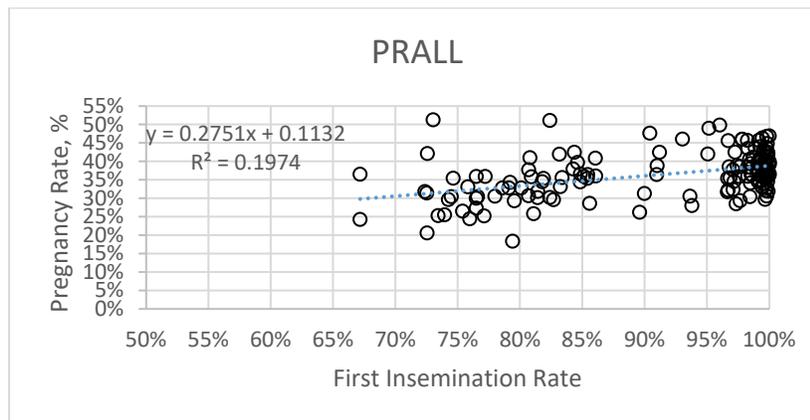


Figure 4b. Pregnancy rate for first insemination rates for 149 herds with excellent reproductive performance across the US. Only one herd with a first insemination rate greater than 75% has a pregnancy rate less than 25%.

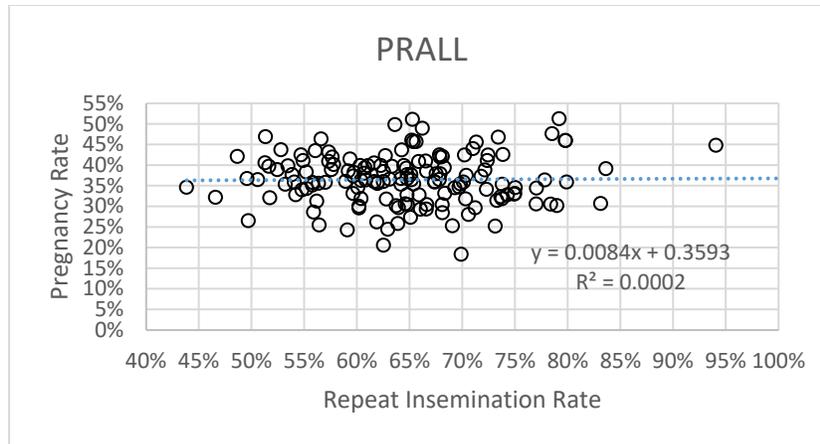


Figure 4c. Pregnancy rate for repeat insemination for 149 herds with excellent reproductive performance from across the US. There is no strong relationship between repeat insemination rates and pregnancy rate, but all most all these herds have repeat insemination rates > 50%.

Based on the observations in Figures 2, 3 and 4, CR is the main driver of PR. Herd management must achieve a $CR \geq 35\%$ to achieve a PR of 25%. Secondly, management must control FSTHDR and achieve rates greater than 70%. Repeat insemination heat detection rate is less critical, but still should be managed to control days between breeding. The average days between inseminations for herds represented in Figure 4 was 32.9 days (+/- 4.4 days). These herds employed excellent management control of reproduction.

Important control points

Two most important control points to achieve a PR of 25% are herd conception rate and first insemination rate.^{1,8} Regressing PR on CR yields an r^2 of 0.70; regressing PR on FSTHDR yields an r^2 of 0.40; regressing PR on RPTHDR yields an r^2 of 0.09. Therefore, priorities are conception, first insemination intensity, then repeat insemination intensity.

Herd conception rate is a function of health postcalving. Cows with any postpartum problem have an odds ratio (OR) of first service conception rate relative to healthy cows of 0.54. Conditions postpartum associated with reduced CR include ovulatory dysfunction, body condition loss of more than $\frac{3}{4}$ of a unit, and infectious and metabolic diseases. Cows that have more than one problem postpartum have a further reduction in first service CR with an OR of 0.36. Approximately, 20 - 30% of problem cows have more than one condition. The average herd experiences about 50% of cows with some health issue postcalving, whereas well-managed herds can reduce this to 30%. It is difficult to eliminate all health issues postcalving. Good nutrition and management practices are necessary to reduce risk of health problems postcalving.

The fertility in healthy cows will determine the CR in a herd, as they will be the most fertile group in the herd. If 50% of cows have no health problems postcalving and CR in these cows is 45%, then herd CR will be 36% and a PR of 25% is achievable. If healthy cows are 50% of animals calving but have a CR of 40%, then herd CR will be 32%, and a PR of 25% is achievable, but is less likely. If CR in healthy cows is 35%, then more than 80% of cows need to have no health problem to achieve a PR of 25%. The take home point is that farm managers need to do all they can to ensure high rates of conception in healthy cows and reduce the proportion of cows with problems postcalving. In addition to cow health, high rates of conception are also dependent on proper time of insemination relative to estrus, good semen handling and placement, and using bulls with good fertility.

Control of first insemination

Reproductive management programs can be structured as follows: chose a VWP that fits the biology of the herd and maximizes CR. Typically 50 - 70 days provides a reasonable range for

establishing a VWP that should be associated with good fertility. Secondly, chose a program to control first insemination intensity. Lastly coordinate veterinary visits and days postinsemination for pregnancy exam to reduce the proportion of cows that are inseminated greater than 48 days from the previous insemination.

Management has some influence on CR. However, management has total control over first insemination intensity and this is the most important management control point for herd reproductive efficiency.^{8,13-15} First insemination intensity (FSTHDR), is the proportion of cows first inseminated in 21 days from the VWP. This is totally under the control of management. To achieve a PR of 25%, FSTHDR needs to be greater than 70%. Figure 3 has the frequency distribution of first inseminations by days in milk for a herd with a PR of 31.3%. This herd has a FSTHDR of 73.1%, mainly performed using visual observation, apparent by the uniform frequency distribution across the 21-day periods. The VWP was 48 days. Conception rate across all inseminations is 44.4%. The combination of intense first service insemination and high CR results in the high PR.

Repeat inseminations are important, but do not have the great impact on PR as CR and FSTHDR. Presented in Figure 3 is the distribution of days between inseminations in categories by day: < 10, 10 - 17, 18 - 24, 25 - 35 and > 48 days. This herd inseminated 58.3% of repeat inseminations between 18 to 24 days following a previous insemination; that represents a regular inter-estrus interval. This is more than double the proportion observed for mean values in Table 1 for herds with PR < 25% and herds with PR \geq 25%. The most critical proportion in this table is the frequency of cows with interval between inseminations \geq 48 days. This herd is only 8.8%, whereas herds with PR less than 25%, this proportion is 29.4%. A significant delay in reinsemination and timely pregnancy examination can reduce this proportion significantly.

There are many ways to control first insemination.⁸ The optimal program for a herd will depend on farm management. Milk progesterone, radio telemetry, and activity monitors require the investment in equipment to improve HDR. Synchronization programs depend on injection of hormones at appropriate timing of the estrus cycle. Visual heat detection requires ancillary aids such as tail paint or pressure sensitive tail patches and 3 - 4 observation periods per day to increase detection to rates greater than 70%. Thus, all programs have some additional cost to control insemination intensity. What is best for any given farm depends on the management structure of the herd.

Synchronization programs

Prostaglandin was first proposed as a program to manage insemination by clustering estruses in cows at specific times.¹³⁻¹⁴ However, depending on day of estrous cycle at the time of prostaglandin injection, cows may not respond to the injection, and time to estrus could vary from 1 to 7 days in cows that seemed to respond to the injection. The variation in time to estrus depended on the stage or follicular development on the ovary, and the day of the estrus cycle of the cow. Sequential injections of prostaglandin could synchronize groups of cows in estrus within weekly periods, but could not synchronize follicular waves, so time to estrus following injection was variable. Therefore, although estrus detection could be focused on specific weekly periods, cows had to be inseminated on observed estrus to have good rates of conception. Cows could be inseminated on a schedule following two injections 11 - 14 days apart, but conception was only optimal if inseminated twice, on days 3 and 4 post injection. Additionally, prostaglandin injections had no benefit to induce estrus in anovulatory cows, which could be 10 - 20% of cows following a VWP of 50 days.

A method using GnRH combined with a prostaglandin injection that synchronized luteal regression and ovulation so timed insemination could be managed on a specific appointment (OvSynch) was proposed.¹⁶ The program consisted of an injection of GnRH to induce ovulation and initiate a new follicular wave. Seven days later, a corpus luteum should be responsive to prostaglandin with an emergent dominant follicle. Following the 7-day prostaglandin injection, 2 days later, a GnRH injection should induce ovulation of a new dominant follicle, that 16 hours later should be fertilizable.^{16,17} Thus, timed artificial insemination (TAI) could be scheduled following the OvSynch program. Initial application in a commercial herd demonstrated the benefit of using this program compared to typical herd management.¹⁸

The OvSynch program induced ovulation in anovular cows. However, fertility in these cows was typically poor. Conception rates are typically 20% in cows entering an OvSynch program that have not previously ovulated. Since anovulatory cows have low serum progesterone, although they may have ovulatory follicles on the ovary, follicles induced to ovulate tend to be more mature and produce lower concentrations of progesterone.^{17,19} Both poorer quality follicles and lower progesterone production reduce fertility in anovular cows.¹⁹ Alternative methods to improve CR in anovular cows have been proposed.

Furthermore, as OvSynch programs were evaluated, it was observed that only 65 to 70% of cows were synchronized, due to several problems with initiating the program in a random group of cows.²⁰ The first GnRH injection of the OvSynch protocol may not induce ovulation due to a regressing or immature follicle on the ovary. When these cows received prostaglandin and GnRH for ovulation and insemination, they tended to have a larger and less fertile follicle than cows that ovulated to the first GnRH injection.²¹ In addition 5 - 15% of cows had only partial luteolysis after the prostaglandin injection, disrupting sperm transport due to elevations in progesterone. Other problems include smaller follicles in a subgroup of cows with insufficient progesterone output postinsemination, in addition to the problem with cows with larger, over-mature follicles. Various additional methods were proposed to improve stage of follicular development and follicular quality prior to the OvSynch schedule. These recommendations included using progesterone intravaginal devices in cows with no CL at the GnRH injection,²² presynchronization with sequential prostaglandin injections prior to OvSynch to increase optimal stage of estrus cycle for synchronization,^{23,24} sequential use of GnRH prior to initiation of OvSynch,²⁵ and combinations of estrus detection following prostaglandin prior to OvSynch.²⁶ Various programs used to control first insemination are shown (Table 2) and various programs used on a group of farms to achieve excellent reproductive performance are detailed.¹⁵ There was no difference in outcome for the various approaches used to control FSTHDR.

Table 2. Programs to control first insemination

1. PGF _{2α}	PGF _{2α}	PGF _{2α}			
	watch estrus 7 days	14 days, watch estrus 7 days			
2. OvSynch	GnRH	PGF _{2α}	GnRH	TAI	
Day	0	7 days	2 days	12 to 16 hours	
3. PreSynch-Ovsynch	PGF _{2α}	PGF _{2α}	OvSynch		
Day	0	14	14		
5. SelectSynch	GnRH	PGF _{2α}	watch estrus	GnRH/TAI	
Day	0	7	72 hours	84 hours after PGF _{2α}	
6. G6G	PGF _{2α}	GnRH	OvSynch		
Day	0	2	8		
7. CIDR7	CIDR	Pull CIDR/PGF _{2α}	Watch Estrus		
Day	0	7	8 - 11		
8. CIDR6	CIDR	PGF _{2α}	Pull CIDR	Watch Estrus	
Day	0	6	7	8 - 11	
9 Anovulatory cows	GnRH	PGF _{2α}	GnRH	OvSynch	
Day	0	7	10	17	
10 No CL at GnRH	GnRH/2CIDR	Pull2CIDR/PGF _{2α}	PGF _{2α}	GnRH/TAI	
	0 of OvSynch	5		6	8

Figure 5 presents distribution of first inseminations associated with various programs. Figure 5a presents first service distribution for a herd using weekly TAI with a Presynch Ovsynch program. Figure 5b presents first service distribution for a herd using a TAI program every 14 days. Figure 5c presents a herd using estrus detection following prostaglandin in the presynch portion of the program than TAI in cows not inseminated in estrus. Distribution of first service reveals the pattern of FSTHDR control employed within a herd. First insemination needs to be greater than 70% to achieve a PR of 25% or better. Conception rate needs to be $\geq 35\%$ to achieve PR of 25% or better.

After first insemination, cows not pregnant need to be reinseminated in a timely fashion. Cows returning in one estrus interval and observed in estrus will be inseminated between 18 to 24 days following a prior insemination. If that estrus is missed, the next opportunity will be 36 to 48 days. With scheduled regular pregnancy diagnosis, nonpregnant cows should be detected between 30 to 40 days postinsemination. Cows diagnosed open could be scheduled into a resynchronization program.

Effect of GnRH injections at 19, 26, and 33 days were compared following insemination with pregnancy examination at 26 days using ultrasonography.²⁷ Open cows then were assigned to a prostaglandin and GnRH TAI program beginning at 26, 33 and 39 days following the initial GnRH.²⁷ The greatest pregnancy at TAI was for cows begun on postsynchronization at 33 days postinsemination. Therefore, resynchronization can be used in cows not re-inseminated by 24 days postinsemination. Double Ovsynch improved synchronization for repeat inseminations compared to 1 OvSynch synchronization.²⁸

Figure 6 presents distribution of days to second insemination for herds using various approaches to control reinsemination. Figure 6a presents the frequency distribution for a herd using visual observation to detect estrus. Distribution is spread over many days, since first insemination is also spread over many days (Figure 5a). Figure 6b is the frequency distribution of days in milk for second insemination in a herd using primarily resynchronization. Cows are clustered from 36 to 48 days for rebreeding. Figure 6c presents distribution of days to second insemination for a herd using visual

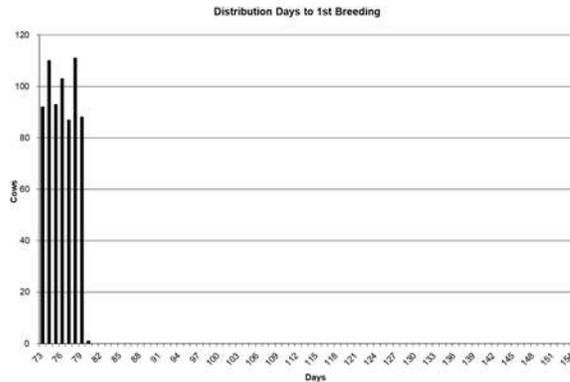


Figure 5a. Distribution of first inseminations in a herd using 100% Presynch Ovsynch every 7 days

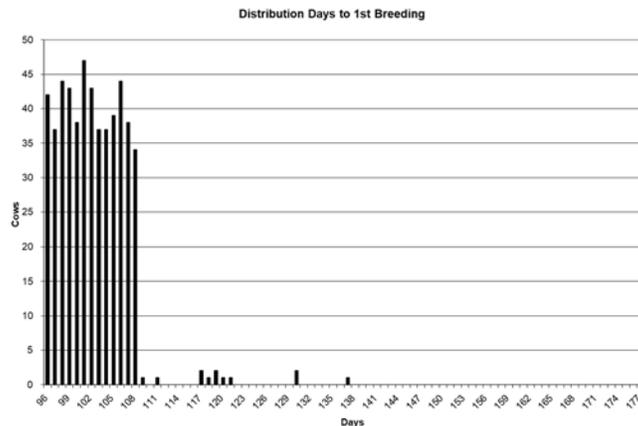


Figure 5b. Distribution of first inseminations in a herd using an OvSynch program every 14 days

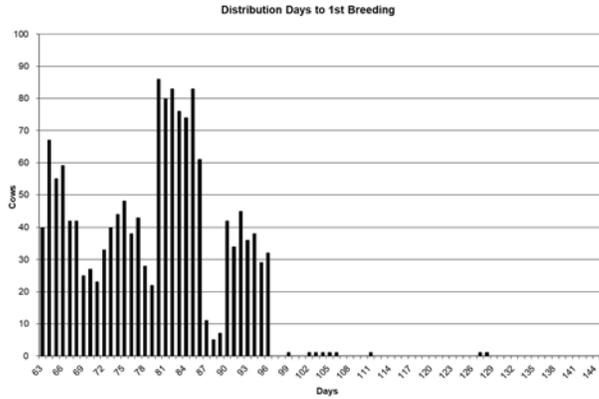


Figure 5c. Herd using estrus detection following prostaglandin Presynch and then applying Ovsynch in cows not inseminated in estrus

observation and then using resynchronization for cows not reinseminated by 30 days postbreeding. Most critical is the herd in Figure 5a has only 8.8% of repeat inseminations over 48 days from the previous service, the herd in Figure 5b has only 4.4% of days between breeding over 48 days, and the herd in Figure 5c has only 7.2% of days between breeding over 48 days. The critical control point for repeat inseminations is a pregnancy diagnosis program to reduce days between breeding over 48 days to less than 10% of inseminations.

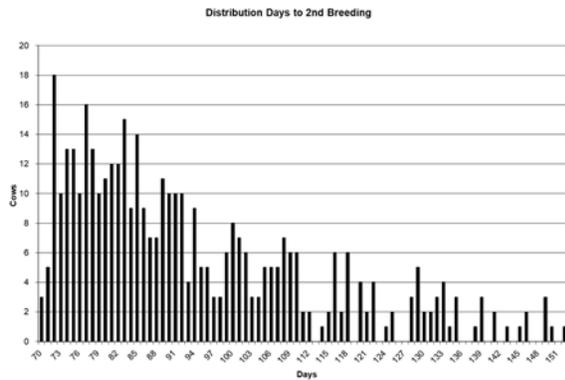


Figure 6a. Herd using visual observation (Herd in figure 3 for first insemination)

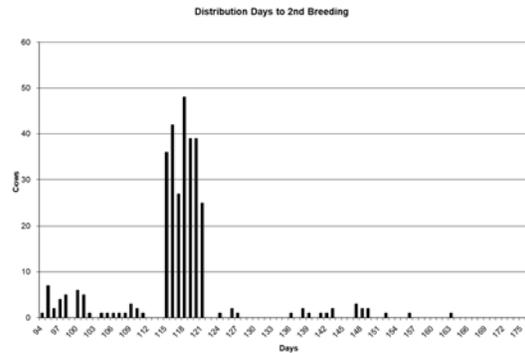


Figure 6b. Herd using resynchronization primarily

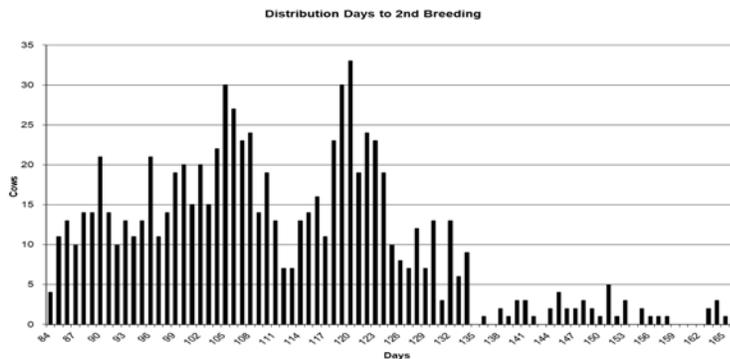


Figure 6c. Herd using heat detection and then re-synchronization

Figure 6. Frequency distribution of days to second insemination for different management programs

Conclusion

Control of first insemination is the critical control point in managing herd reproduction. Monitoring fertility in healthy cows establishes baseline fertility in the herd and provides an estimate of the wellbeing of transition cows and insemination protocols. Healthy cows should have FSTCR greater than 50%. First service intensity needs to be greater than 70% and pregnancy performed on a timely basis before 40 days postinsemination. Many opportunities exist to control first insemination. The best strategy will depend on herd management. Automatic activity monitoring versus synchronized breeding programs could not be generalized, but depended on management within each herd.²⁹

Conflict of interest

There are no conflicts of interest to declare.

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Reproductive immunology and endocrinology of pregnant mare

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Abstract

Pregnancy is a distinct event that induces a unique relationship between immune and endocrine systems. In humans, there is growing evidence that pregnancy is regulated by both immune-immune and immune-endocrine interactions, and that these are constantly shifting throughout pregnancy. Pregnancy-related hormones (progesterone, estrogen, and human chorionic gonadotropin) are critically involved in successful establishment, maintenance, and termination of pregnancy. In humans, these hormones suppress detrimental maternal alloresponses to semi-allogeneic fetus while promoting tolerance pathways. Although there is a better understanding of endocrinology of equine pregnancy, minimal inferences are made understanding immune-endocrine interactions. Our current understanding of endocrinology and immunology of equine pregnancy is reviewed and advancements needed to properly identify interactions between these 2 systems are provided.

Keywords: Mare, pregnancy, immunology, endocrinology, fetus

Reproductive endocrinology of pregnant mare

Progestins

Progesterone is necessary for maintenance of pregnancy in mammals, but mare is somewhat unique, as source and type of progestin varies during pregnancy.¹ This aspect of endocrinology of equine pregnancy has been known since mid-1950s when Short demonstrated that progesterone could not be measured in blood of mares after mid-pregnancy.² Advent of mass spectrometry coupled with chromatography provided a more specific analysis of individual progestins in blood required to further characterize progestins present in circulation during latter half of equine pregnancy.³ This study identified 5 α dihydroprogesterone (DHP) and its metabolites as major pregnanes present in circulation of mares during second half of pregnancy. Relative bioactivity of DHP in mare remained speculative until studies demonstrated DHP alone could maintain pregnancy in mares in progesterone absence and that DHP and progesterone had equal potency in binding to equine progesterone receptor (PR).⁴ Interestingly, equine PR has a mutation in progesterone binding region similar to elephant, another species that utilizes DHP for pregnancy maintenance.⁵

Initial source of progesterone for pregnancy maintenance in mares is corpus luteum. In addition to progesterone, DHP is present during early pregnancy (also in nonpregnant luteal phase) in concentrations ~ 50% of that of progesterone in circulation.^{4,6} Source of DHP during early luteal phase is uncertain; however, it appears that luteal progesterone is rapidly metabolized to DHP through activity of 5 α reductase type II,⁶ which is highly expressed in skin.⁷ Beginning near days 35 - 37 of pregnancy, secretion of equine chorionic gonadotropin (eCG) by endometrial cups initiates an increase in circulating progestins (both DHP and P₄) from primary corpus luteum (CL) and from newly formed secondary CLs in pregnant mare. As chorioallantois develops more fully, placental synthesis of progestins increases after day 70 of pregnancy, and concentrations of DHP exceed those of P₄ ~ 105 days of pregnancy.^{8,9} This transition has been termed luteal-placental shift in progestin synthesis,¹⁰ characterized by inputs from both fetus and placenta for progestin synthesis.^{3,9,11} Feto-placental synthesis of progestins involves synthesis of progrenolone (a universal precursor in steroidogenesis) that is subsequently converted to DHP by

5 α reductase (Figure 1) expressed in chorioallantois.⁹ A number of metabolites of DHP are detected in circulation of pregnant mares after mid-pregnancy, some of which are present in very high concentrations as gestation progresses.^{3,8} Biological function of these DHP metabolites remains uncertain, although some may have a role in activating PR, because despite their low affinity for PR, they are present in such high concentrations.¹²

Withdrawal of progestogenic support at end of pregnancy is an important mechanism in parturition initiation in most species studied. Data related to a reduction of progestins prior to parturition in mares have been conflicted, with some but not all studies identifying changes detected prior to foaling.^{1,10} More recent research has

identified a decline in DHP and its associated metabolites 3 days preceding parturition in mares.^{8,12} This decline in 5 α reduced progestins in days preceding foaling is associated with marked reduction in expression and enzymatic activity of 5 α reductase in chorioallantois.¹³ Although mechanism regulating this downregulation of 5 α reductase is unknown, this appears to be a key event in preparing the endocrine environment for onset of parturition.

Monitoring progesterone concentrations (and its 5 α reduced metabolites) has been used for many years to evaluate well-being of equine pregnancies in both experimental and field studies.¹⁴⁻¹⁸ Interpretation of these studies is sometimes confusing and is hindered by use of immunoassays for measurement of progesterone concentrations, with variable cross-reactivities to pregnanes, some of which may be present in very high concentrations.¹⁹ Application of mass spectrometry has been helpful to understand changes in pregnanes in both normal and abnormal equine pregnancy.^{3,18,20} More chronic pathology of placenta (e.g. ascending placentitis) of late pregnancy is associated with a marked increase (3 - 4 fold; Figure 2) in 5 α dihydroprogesterone (DHP), along with several of its metabolites.^{18,20} Although concentrations of P₄ also change based upon liquid chromatography-mass spectrometry (LC-MS), P₄ concentrations remain relatively low; therefore, most studies measuring changes in progestins with immunoassays were likely detecting changes in 5 α reduced progestins (present in vastly higher concentrations). Interpretation of maternal serum pregnane concentrations becomes more difficult after day 300 of pregnancy, due to normal prepartum rise in pregnanes that occurs at this time⁸ and because pregnane concentrations become more variable as parturition approaches.

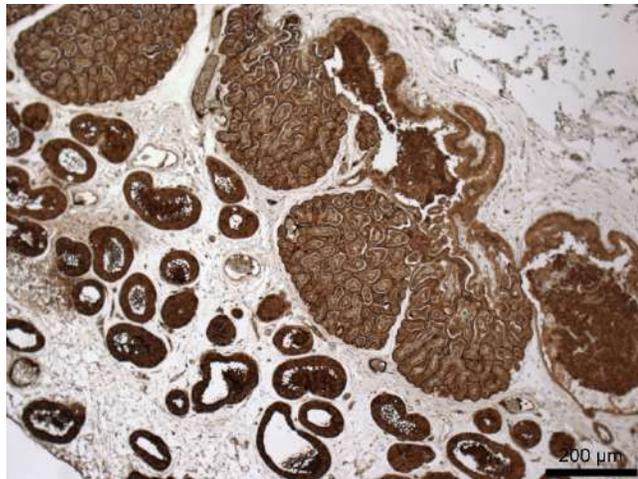


Figure 1. Immunohistochemical labeling of 5 α reductase in equine placenta at day 300 of pregnancy. Both chorionic and endometrial epithelial cells have distinct (brown) labeling.

In contrast to chronic placental disease, more acute placental infections appear to be associated with a rapid decline in maternal serum pregnane concentrations that precede abortion by ~ 3 days.¹⁸ At endometrium and chorioallantois, tissue concentrations of 5α DHP and its metabolites are decreased in acute inflammation.²¹ This reduction in local concentrations of pregnanes is associated with a downregulation of key steroidogenic enzymes (5α reductase) which is likely responsible for both tissue and systemic decrease in pregnanes in these acute placental lesions.

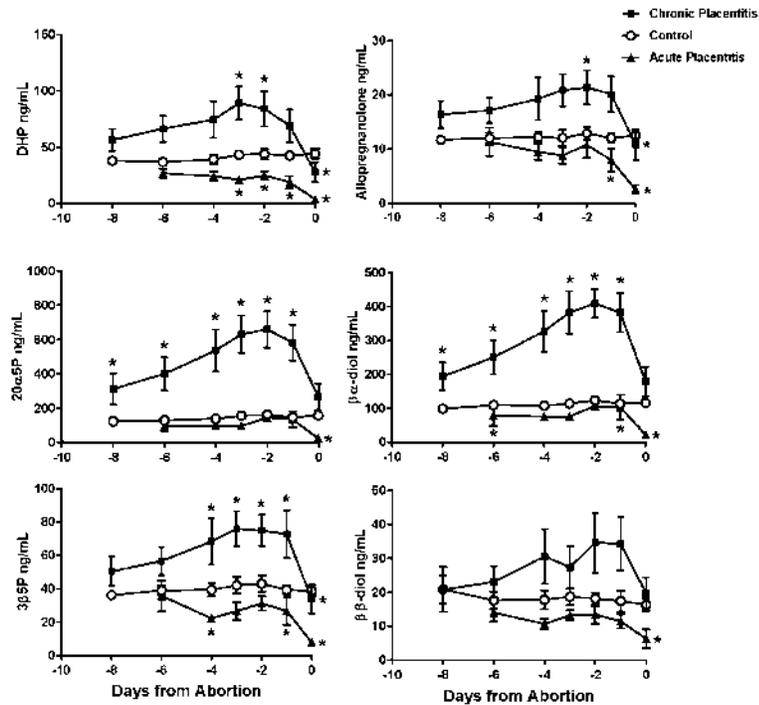


Figure 2. Plasma concentrations of pregnanes (5α dihydroprogesterone (DHP), allopregnanolone, 20α hydroxy 5α pregnan 3 one (20α 5P), 5α pregnan 3β , 20α diol ($\beta\alpha$ diol), and 3β hydroxy 5α pregnan 20 one (3β 5P), and 5α pregnan 3β , 20β diol ($\beta\beta$ diol)) measured by liquid chromatography tandem mass spectrometry LC-MS/MS. Reprinted (Theriogenology 2018;122:130-136.) with permission.

Estrogens

Elevated estrogen concentrations in peripheral circulation are a hallmark of equine pregnancy and have been described since the 1930s.²² Increased estrogens concentrations may be detected in early pregnancy from corpus luteum, associated with eCG secretion.²³⁻²⁵ Again, as fetoplacental unit becomes more active in steroidogenesis, placenta takes over estrogen secretions. In this case, secretion of androgens (dehydroepiandrosterone, DHEA) by fetal gonads serve as a precursor for aromatization by placenta, through aromatase action (Figure 3), which is highly expressed in chorioallantois.¹ Circulating maternal estrogens peak at ~ 7 months, which corresponds to a marked hypertrophy of fetal gonads (both ovary and testis) associated with growth of interstitial tissue.

Exact function of elevated estrogens during equine pregnancy is not fully understood. Inhibition of estrogen synthesis by aromatase during months 2 - 4 reduced fetal growth, reduced expression of angiogenic genes and reduced vascular development in endometrium (Haneda and Ball, unpublished). Inhibition of aromatase activity by administration of letrozole during last trimester of equine pregnancy decreased maternal concentrations of 17β estradiol and estrone sulfate by 90% compared to controls.²⁶ Uterine blood flow (measured in uterine artery by Doppler ultrasonography) was not affected by

decreased maternal estrogens and pregnancy length and neonatal viability was similarly not different when estrogens were markedly suppressed.²⁶ Birthweights were reduced by ~ 15% in estrogen-suppressed mares, and this effect may have been due to effects of uteroplacental microvasculature that were not detected as changes in uterine artery blood flow.

Elevations in maternal estrogens are classically associated with initiation of parturition in domestic ruminants. Although there is no prepartum increase in circulating estrogens in peripartum mares, there is a sustained estrogen secretion and aromatase activity in chorioallantois remains elevated through parturition in mare.¹³

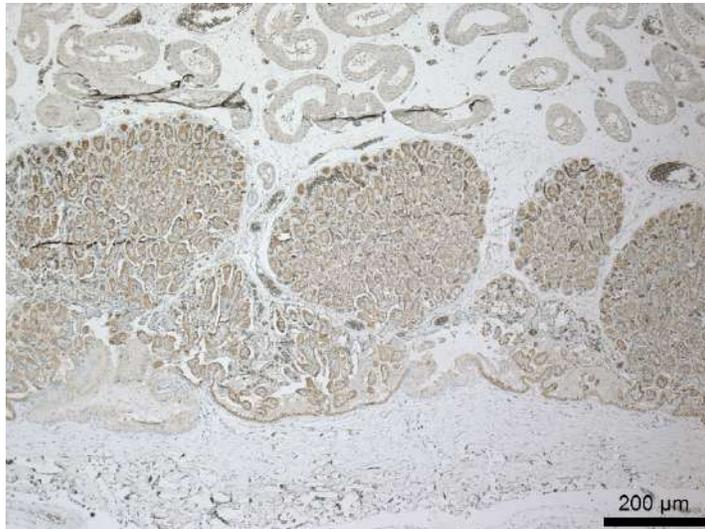


Figure 3. Immunolabelling of aromatase in day 300 equine placenta. Aromatase is expressed primarily in chorion epithelial cells and chorionic villi.

Maternal concentrations of estrogens have also been used to assess fetal wellbeing.^{17,27-29} Reductions in 17β estradiol appear to be more closely indicative of fetoplacental endocrine function than changes in estrone sulfate,²⁹ possibly because of relatively longer half-life of sulfo-conjugated estrone. In experimentally induced ascending placentitis, 17β estradiol concentrations declined ~ 6 days preceding abortion.²⁹ Similarly, mares with fetal loss had a lower 17β estradiol concentration than mares that produced a healthy foal in a field study of 459 pregnant mares.¹⁷ These changes in peripheral estrogen concentrations in mares with placental compromise appeared to be associated with a decreased expression of aromatase and a reduced tissue concentration of estrogens in mares with placental infection²¹.

Activins and inhibins

Although inhibins and activins are most commonly thought of as gonadal hormones, elevations in inhibins and activins have been reported during pregnancy in women;³⁰ however, studies on changes in these hormones during equine pregnancy are contradictory. Higher concentrations of immunoreactive inhibin in circulation of mares during later pregnancy originated from fetal gonads.³¹ This immunoreactive inhibin was not biologically active and assay did not distinguish different forms of inhibin. Specific immunoassays detected decreased inhibin A and inhibin B at 60 days after ovulation and lower amounts until end of pregnancy.³² Molecular characterization of inhibin/activin subunits from the endometrial and fetal placenta suggested that inhibin/activin A was primary isoform present.³³ This suggested that activins rather than inhibins were predominant form in pregnant mare uterus.

Activins belong to transforming growth factor β superfamily. Activins have a common β subunit and are homodimers of β with α isoforms (A, B, and AB). Induction of experimental placentitis in mares ~ 280 days of pregnancy resulted in an increase in activin A in maternal circulation, along with an increase in activin A in allantoic fluid of mares with placentitis compared to gestationally age matched control mares.³⁴ Activin A has been described as an acute mediator of inflammation in horses and other species. Although source of increased activin A in mares with induced placentitis is unknown, it is likely derived from the uteroplacental unit. Future studies should address the utility of activin A determination in clinical cases of placentitis.

Reproductive immunology of pregnant mare

Field of reproductive immunology in humans has advanced considerably in the 70 years since Sir Peter Medawar stated his initial hypotheses for how the maternal immune system supports a semi-allogeneic fetus. In 1953, Father of Transplantation stated “The immunological problem of pregnancy may be formulated thus: how does pregnant mother contrive to nourish within itself, for many weeks or months, a foetus that is an antigenically foreign body?” Yet through inventive research on his part alongside others, it is now understood that pregnancy is controlled by both innate and adaptive system, and that fetus develops under a state of active tolerance. Additionally, pregnancy cannot be considered a single event, and different stages of pregnancy require immensely different immunophenotypes. Recent research indicates that this accomplished through a series of immune-immune interactions and immune-endocrine interactions that create a complex network of immune regulation to ensure fetal survival. Various hormones that are upregulated in pregnancy have profound effects on the immune system; this includes progestins, estrogens, and human chorionic gonadotropin (hCG). Equine pregnancy differs from humans’ in a multitude of ways, including placentation, implantation, and endocrinology and therefore inferences are difficult to make. Here we review our current knowledge of this process in the horse.

Immediately after deposition of foreign and allogeneic semen into uterine lumen, innate immune system is activated through a series of proinflammatory mediators, leading to the chemotaxis of neutrophils, macrophages, and dendritic cells.^{35,36} Neutrophils are present within 30 minutes following breeding, are at the highest numbers at 12 hours, and undetected by 24 hours after breeding in the normal horse and assist in the clearance and digestion of excess spermatozoa and contaminants.^{37,38} Anti-inflammatory cytokines IL10 and IL1RN are elevated < 6 hours postbreeding, signaling for innate response resolution and persistent inflammation suppression.³⁵ In other species, maternal uterine dendritic cells bind to paternal antigens present in seminal fluid before migrating to lymph nodes to activate adaptive immune system to recognize seminal antigens as self, and this is specifically performed by T cells.³⁹⁻⁴³ This has not been confirmed in mare.⁴⁴ T cells are key regulators of adaptive immune response to pregnancy and can be divided into numerous subsets, including helper T cells (Th1, Th2, Th9, Th17, Th22), regulatory T cells (Tregs), and cytotoxic T cells (CTL).⁴⁵

Both CD4+ cells, Th1 and Th2 cells develop following antigen presentation through a series of interactions with specific cytokines, transcription factors, and hormones. Th1, or IFN γ secreting cells, are proinflammatory and are required for response to intracellular pathogens. In contrast, Th2, or IL4-secreting cells, are anti-inflammatory and necessary for response to extracellular pathogens. In humans, estrogen receptor (ER) is found on both CD4+ and CD8+ cells, and estrogen has been shown to decrease both Th1 and Th2 cytokines TNF and IL4.⁴⁶ In contrast, progesterone inhibits Th1 secreted IFN γ ,⁴⁷ whereas also promoting Th2 secreted IL4.⁴⁸ Studies from our lab identified in diestrus mare decreased endometrial expression of proinflammatory IFN γ , IL1 β , and IL8 in comparison to day of ovulation, indicating an anti-inflammatory effect of progesterone in mare.⁴⁹ Due to progesterone-dependency of

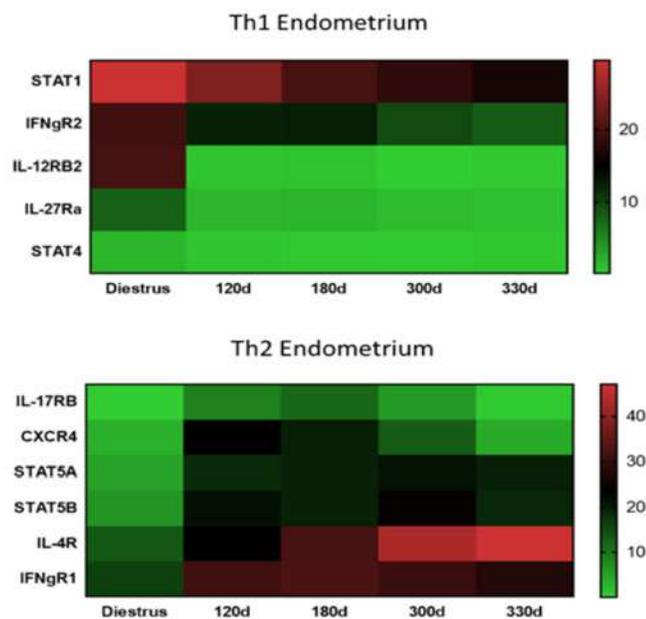


Figure 4. Heat map of Th1 and Th2 related transcripts altering during equine pregnancy. Red indicates higher transcript expression levels; green indicates lower transcript expression levels

Th2 cells,⁴⁸ lymphocyte subset was initially believed to regulate pregnancy maintenance and prevent rejection of the semiallogeneic tissues (both fetal and chorion).⁵⁰⁻⁵⁶ It is now understood that the ratio of Th1:Th2 is in constant fluctuation throughout pregnancy, with implantation and parturition being primarily Th1 events, whereas mid-pregnancy is shifted towards a Th2 environment. In mare, this relationship was similar to that previously described in humans. Th1 related transcripts at the fetomaternal interface were highest in diestrus endometrium and decreased as pregnancy advanced. In contrast, Th2 transcripts increased in endometrium to mid-pregnancy and declined prepartum (Figure 4).⁵⁷ Interestingly, no Th1 related transcripts were altered during equine pregnancy in chorioallantois, whereas Th2 transcripts followed a similar profile to that noted in the endometrium.

Recently, immunology of pregnancy was determined to be less controlled by the Th1:Th2 ratio, and more so by regulatory T cells (Tregs). Paternal antigens are present within semen,⁴² and signal expansion of Tregs to identify this antigen as self and not requiring attack.⁵⁸ Tregs are believed to be the key regulator of active state of tolerance during pregnancy. Estrogen causes a dose-dependent increase in Treg secreted IL10 and Foxp3+ Tregs that are increased in estrus in comparison to diestrus, indicating estrogen dependency.⁵⁹ Interestingly, recent research in a murine model identified activin induced production of Foxp3 gene and IL10 secreting Tregs.⁶⁰ These cells are highly immunosuppressive and inhibit production of Th2 cell types. Additionally, inhibin-null mice (*Inhα^{-/-}*) had increased production of Tregs in periphery, indicating endocrine involvement in Treg development.⁶¹ In humans, Tregs are lowest during implantation, increase towards mid-pregnancy and decline prepartum, following a similar profile to that of estrogen production during pregnancy.⁶⁰⁻⁶² In mare, a similar profile was noted in Treg-related transcripts at fetal maternal interface.⁵⁷ Treg-related transcripts were higher in pregnancy compared to the nonpregnant endometrium; this profile was also noted in the chorioallantois. Additionally, transcript was expressed lowest in early pregnancy, increased towards mid-pregnancy and declined prepartum, also following estrogen profile production during equine pregnancy (Figure 5).

It should be noted that comparative physiology between equine and human pregnancy indicates many differences, and therefore inferences are difficult. Human implantation occurs on day 9 following fertilization, whereas equine pregnancy does not have a true implantation event until day 35 when the eCG-secreting endometrial cups develop from the chorionic girdle.⁶²⁻⁶⁴ Invasion of the trophoblast into the endometrium leads to a rapid influx of maternal lymphocytes, including CD4+ and CD8+ T cells, but their activity remains suppressed until ~ 80 days of gestation, at which point they actively attack and degrade the cups.^{65,66} Ratio of lymphocytes within the endometrial cup is predominantly Th1, implying that a heightened Th1 response may be necessary for their degradation, although the signaling required is unknown.⁶⁷ The regression of the cups leads to a decrease in eCG production, and a subsequent decrease in ovarian-produced progesterone.⁸ This coincides with an increase in the fetal placental progestins, including 5 α DHP and 20 α 5P. Similar to eCG, early human pregnancy has high hCG concentrations. Little is known about the role of eCG on the immune system, but it was recently reported that hCG affects the immune response in a variety of ways, including proliferation of uterine natural killer cells,⁶⁸ increased monocytic function,⁶⁹ inhibition of Th1 development,⁷⁰ and recruitment and activation of Tregs.⁷¹ Although effects of eCG on immune function in mare has not been

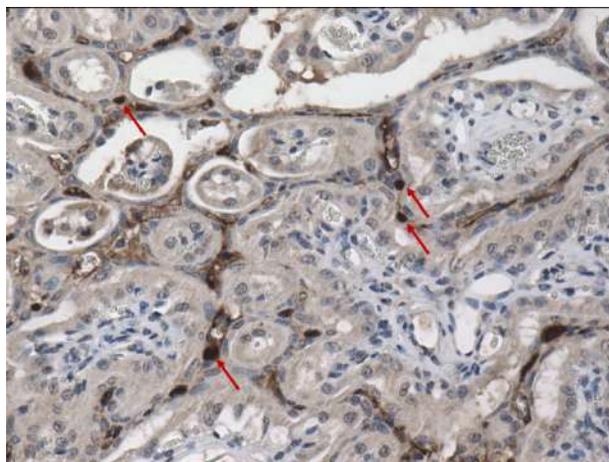


Figure 5. Foxp3+ Tregs in the 300d intact placenta. Arrows indicate positive immunolabeling (brown). Reprinted from (Placenta 2020;89:78-87) with permission.

well investigated, it is known that placental explants containing eCG were immunosuppressive and inhibited proliferation of lymphocytes (specific cell type remains to be determined).⁷²

Pregnancy complications can lead to alterations in normal endocrine-immune response. As noted, fetal compromise is associated with shifting endocrine profiles, including a decrease in estrogens and an alteration in progesterin concentration dependent on disease progression nature. Immunologically, abortion-prone women tend to experience an increase in Th1 cell populations in circulation, in addition to having increased concentrations of Th1-secreted IL2 and TNF, although Th2 immunophenotypes have also been noted in women suffering recurrent miscarriage.^{51,54} Interestingly, a deletion of either cell type does not lead to abortion or infertility, indicating lack of reliance for pregnancy maintenance. In contrast, a deletion of Tregs leads to both abortion and implantation failure,⁷³ whereas a decrease in estrogen Tregs is associated with a variety of pregnancy complications, including pre-eclampsia, chorioamnionitis, repeat miscarriage, and preterm labor.^{58,74-77} In the horse, a decrease in Tregs is associated with early embryonic loss, although less is understood in late gestation.⁷⁸ Data from our lab (unpublished) suggests that ascending placentitis is associated with an increase in Th1-related transcripts and dysregulation of Th2 and Treg-related transcripts at the feto-maternal interface, but less is known in the periphery. Additionally, it appears that the chorioallantois is the primary responder to induced infection and undergoes upregulation of Th1-related transcripts following infection. It is unknown if this alteration in Tregs-related transcripts is associated with the systematic decline in estrogens noted during fetal compromise in the horse, but this warrants additional research.

Conclusion

Pregnancy is a distinct event that induces a unique relationship between immune and endocrine systems. How maternal immune system tolerates the semi-allogeneic fetus has fascinated researchers for centuries. Recent research indicates that this is accomplished through a series of immune-immune interactions and immune-endocrine interactions that create a complex network of immune regulation to ensure fetal survival. Various hormones that are upregulated in pregnancy had profound effects on immune system; this includes progesterone, estradiol, and human chorionic gonadotropin, although few of these interactions have been confirmed in mare. It is imperative that we improve our understanding of this relationship in normal equine pregnancy before making inferences into alterations of the endocrine-immune interaction of the abnormal.

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Conflict of interest

None to report.

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Genomics, transcriptomics, and proteomics of normal and abnormal equine placenta: better understanding of late pregnancy function and dysfunction

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Abstract

High-throughput methods to assess genomics, gene messenger RNA expression, and protein composition of tissues and body fluids have led to rapid advancement in understanding of normal and abnormal function of many body systems across a wide variety of animal (mammalian and nonmammalian) species. Over several years, these techniques were applied to study normal physiology and disease of pregnant mare, focusing on placental and fetal fluids. Although our understanding of endocrine aspects of pregnancy in mares is reasonably advanced, much of our understanding related to placental function and dysfunction remains limited. This review covers studies that detailed normal pregnancy changes in fetal and maternal placenta, along with changes in gene expression in a number of late-pregnancy diseases.

Keywords: Equine, placenta, transcriptome, placentitis, pregnancy

Introduction

Advent of high-dimensional biological techniques, including genomics, transcriptomics, proteomics, lipidomics, and metabolomics and increasing application of these techniques marks a landmark in research to understand normal physiologic function and pathophysiology of disease in animals. In particular, high-throughput RNA sequencing (RNA-Seq) allows quantitative evaluation of gene expression in a tissue or cell and investigation of different isoforms of transcript present. These approaches generate massive datasets that require high-capacity computing platforms for bioinformatics analysis. Fortunately, equine genome sequencing¹ and subsequent updates to the equine genome (EquCab3.0)² provided detailed information on equine genome. This information, in combination with information overload regarding gene structure and function, cellular, biological, and disease pathways from biomedical research in humans and laboratory species, heralded a new era in veterinary medical research. Objective is to summarize recent studies in authors' laboratory using these approaches to better understand normal physiology and pregnancy disease in mare.

Transcriptomics of normal fetal and maternal placenta across pregnancy

Much research on chorioallantois (CA) and endometrium (EN) as fetal and maternal portions of placenta in domestic animals has focused on early pregnancy (maternal recognition of pregnancy) or very late pre-term changes. We examined changes in messenger RNA (mRNA) transcripts in both CA and EN across pregnancy (1.5, 4, 6, 10, and 11 months) in mare.³ Large number of differentially expressed genes (DGE) were identified, with 5,932 and 3,667 DEG in CA and EN, respectively. Greatest difference in expression occurred at 4 or 11 months of pregnancy. Unsurprisingly, most highly expressed genes in CA and EN were related to either endocrine or immune function. Highly expressed genes included endocrine-related transcripts (RLN, CYP19A1, HSD3B2, SPP1, PLA2G10, INHBA), immune-related transcripts (CST3, CTSL, SERPINA3, SERPINA6, SERPINA14, SPINK7, SPINK9, LTF, S100A6, SLPI), iron binding proteins (ACP5, FTH1, HBA2, LCN2, SERPINA14), and serine protease inhibitors (SERPINA3, SERPINA6, SERPINA14, SPINK7, SPINK9). Others included extracellular matrix proteins (ECM1, SPARC, MMP26), transport proteins (ACP5, GM2A, HBA2, LCN2), and antioxidants (PRDX1, SOD3). Evaluation of gene expression networks provides ability to examine cellular or biological pathways associated with changes in transcript abundance. In fetal and maternal placenta, many pathways associated with cell growth, mitosis, metabolism, oxidative stress, angiogenesis, and steroidogenesis were upregulated, whereas immune-related pathways, including B cell activation, leukocyte and lymphocyte activation and immune response, were downregulated. These findings are consistent with fetal placenta

needs for continued growth, steroid synthesis, and transport of materials to fetus, along with protecting allogeneic fetus from maternal immune response.

Analysis of placental transcriptome across pregnancy has also been useful in better understanding endocrine function of both EN and CA during equine pregnancy.⁴ In particular, evaluation of various isoforms of steroidogenic enzymes in CA and EN across pregnancy reveals close coordination between these 2 tissues for pregnane and estrogen synthesis regulation during pregnancy. Similarly, changes in steroid receptors during pregnancy imply differences in relative importance of receptor types during pregnancy. Nuclear progesterone receptor has lower and relatively constant expression in CA and EN for most of pregnancy; however, membrane associated progesterone receptor (PGRMC1) is highly expressed in EN and CA, which suggest that placental effects of pregnanes in mares may be mediated by these receptors in mares.⁴

MicroRNA in chorioallantois, endometrium and circulation in normal and abnormal equine pregnancy

In addition to protein coding messenger RNA (mRNA), there are a variety of small noncoding RNA (ncRNA) that are detected with RNA-Seq. These ncRNA appear to regulate expression of mRNA and provide additional information about gene function. Of ncRNA, microRNA (miRNA) are small (20 - 22 nucleotides) RNA that regulate protein coding genes. Expression of miRNA clusters have been described in human placenta, including human chromosome 14 (C14MC) that appears to be highly conserved across eutherian mammals and seems to have an important role in placental development.⁵ In horse, orthologous miRNA cluster is located on ECA24 (C24MC).⁶ Expression of miRNA in C24MC in equine placenta was higher in earlier pregnancy, but declined with advancing pregnancy.⁶ Target mRNA of miRNA in C24MC cluster had a reciprocal expression pattern (increased with pregnancy), and many transcripts regulated by these miRNAs were related to angiogenesis and vascularization of placenta with advancing pregnancy.⁶

Unlike mRNA, miRNA have a relatively long half-life in circulation, and changes in circulating miRNA have been identified in normal and abnormal pregnancy in animals and in humans, with potential application as diagnostic biomarkers.⁷⁻¹⁰ In an initial, PCR-based study, we identified 1 miRNA that was differentially expressed in late pregnancy (miR-374b) and 4 miRNA that were differentially expressed during pregnancy in mares (miR-454, miR-133b, miR-486-5p, and miR-204b).¹¹ These pregnancy-specific miRNA targeted pathways related to placentation, angiogenesis, and endocrine function during pregnancy.¹¹ Members of C24MC cluster of miRNA were also detected in circulation of pregnant mares.¹² MicroRNA from C24MC cluster were more highly expressed in circulation during early pregnancy, consistent with their expression pattern in placenta. Serum enrichment with miR-1247-3p, miR-134-5p, miR-382-5p, and miR-433-3p at day 25 pregnancy and miR-1247-3p, miR-134-5p, miR-409-3p, and miR-379-5p at day 45 pregnancy suggest that these miRNAs are involved in early pregnancy events.¹²

One goal of examining miRNA expression in serum during pregnancy was to evaluate use of miRNA in blood as potential biomarkers during abnormal equine pregnancy. For this study, RNA-Seq was used to screen ncRNA expression in CA, EN and blood of mares with experimentally induced placentitis at ~ 280 days of pregnancy.¹³ Tissues collected between 3 - 5 days after inoculation in treated mares and uninoculated mares of comparable pregnancy used as controls.¹³ Analysis of ncRNA expression in blood, CA and EN revealed 658 and 507 miRNA for tissue and blood, respectively. Principal component analysis of these data revealed distinct clustering of samples based upon tissue of origin and disease state. A total of 50 ncRNA were differentially expressed between control and placentitis tissue samples. Differentially expressed miRNA included 26 in CA, 20 in EN and 9 in serum. Of 9 miRNA that changed in serum, 6 also exhibited parallel changes in either CA or EN.¹³ Many miRNA that were upregulated in equine placentitis were also upregulated in women with chorioamnionitis, suggesting that aspects of these disease processes are conserved. Many miRNA that were dysregulated in equine placentitis were associated with altered immune function, in particular, regulation of inflammation mediating cytokines IL6 and IL8, as well as activation of macrophages and

lymphocytes. Whether or not changes identified in circulating miRNA in mares with experimental placentitis will have utility in diagnosis of spontaneous equine placentitis remains to be determined. Although different changes detected between controls and treated mares, magnitude of changes may not lend themselves to good diagnostic tests, and it is likely that a panel of miRNA will need to be evaluated as possible biomarkers.

Changes in transcriptome during abnormal equine pregnancy

Nocardioform placentitis

Nocardioform placentitis (NP) remains a poorly understood disease of placenta in mares. It is characterized by late-term abortions and fetal growth retardation associated with a distinct placental lesion located typically at ventral aspect of placenta, distinct from cervical star. NP is associated with gram-positive, branching actinomycetes including *Amycolatopsis* spp., *Crossiella equi* along with more recently characterized isolates of *Streptomyces atiruber* and *Streptomyces silaceus*.¹⁴⁻¹⁶ During 2017 foaling season, we collected placenta from mares suspect for nocardioform placentitis (n = 4) and 4 normal placentas as controls. RNA isolated from these tissues was analyzed by RNA-Seq.¹⁷ A total of over 3,000 genes were differentially expressed in placenta from mares with NP. Signaling pathways related to inflammation (cytokines and chemokines), pattern recognition receptors (toll-like receptors), apoptosis (caspases), hypoxia, angiogenesis and antimicrobial peptides upregulated in placenta from mares with NP were compared to normal term placenta.

Ascending bacterial placentitis

In addition to nocardioform placentitis, ascending bacterial placentitis remains an important cause of late-term pregnancy loss in mares. We examined changes in transcriptome of both CA and EN recovered from mares with experimentally induced placentitis (*Streptococcus equi* spp. *zooepidemicus*) at 8 days after initial transcervical inoculation and ~ 290 days of pregnancy. Uninoculated mares served as controls; ~ 3000 genes were differentially expressed in CA and ~ 1000 DEG were detected in EN from mares with ascending placentitis. Upregulated pathways in CA included inflammation, interleukin and integrin signaling, angiogenesis, apoptosis, toll-receptor signaling, and B cell and T cell activation (El-Sheikh and Ball, unpublished). Upregulated pathways in EN included inflammation and integrin signaling, and toll receptor signaling. These changes, in turn, were associated with dysregulation of placental steroidogenesis, angiogenesis, nutrient transport, and hypoxia. A number of matrix metalloproteases were also upregulated in CA that may be associated with degradation of extracellular matrix and resultant placental separation. Related pathways and mechanisms associated with this dataset are illustrated (Figure 1).

Premature placental separation

Premature placental separation (PPS; red bag placenta) is a common and poorly understood problem in foaling mare. Premature separation of CA from endometrium without rupture of CA at cervical star during late first stage and second stage labor may result in significant fetal hypoxia if not identified quickly and corrected by opening chorioallantois to assist foal delivery. Incidence of PPS varies with study, but cited as 1.6% of 1,047 foaling with a mortality rate of 17.6%,¹⁸ 0.9% of abortions presented in Normandy France¹⁹ or 4.7% of reproductive losses in central Kentucky.²⁰ During late abortions associated with Mare Reproductive Loss Syndrome (MRLS), incidence of PPS was reported as 28% of cases.²¹ These data likely underestimate PPS frequency, because many such cases are likely not presented to diagnostic laboratories if neonate is not overtly affected during delivery. Clinically, PPS has been variably associated with problems such as endophyte-infected fescue,²² placental inflammation associated with viral (EHV 1) placentitis,²³ and ascending bacterial placentitis.²⁴ Although clinical presentation of equine PPS is well known, underlying pathophysiology of problem in mare is poorly understood. Therefore, we examined holistic changes in gene expression in CA of mares with premature placental separation using next generation sequencing technology. We performed RNA-Seq on CA from

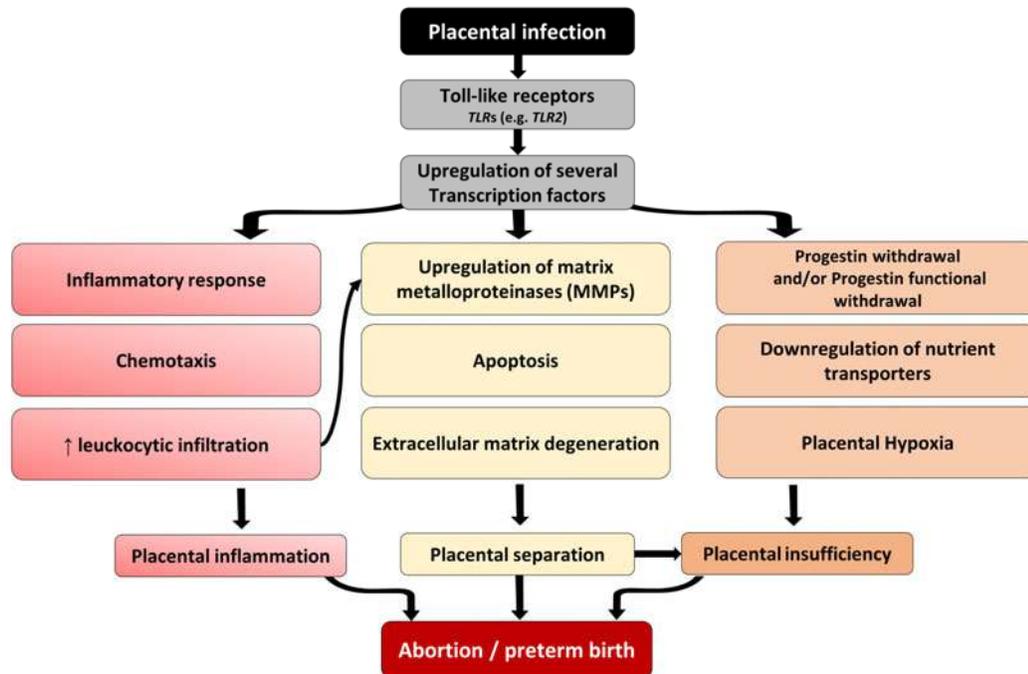


Figure 1. Schematic illustration of possible mechanisms associated with ascending placentitis in mare.

mares with PPS (n = 33) and mares with normal parturition as controls (n = 4). Resulting RNA-Seq data were subjected to standard analysis pipeline to examine differentially expressed genes and associated pathways and upstream regulators (Murase, El Sheikh Ali and Ball, unpublished data). A large number of differentially expressed genes (DGE) were identified with 5,932 and 3,667 DEG in CA and EN, respectively. A number of genes associated with extracellular matrix, including collagens, proteoglycans, and metalloproteinase inhibitors were upregulated in mares with PPS compared to control CA. Key upstream regulators identified include transcripts associated with hypoxia, inflammation, extracellular matrix, and cell adhesion (Figure 2).

Hydrops allantois

Hydrops conditions are rare in mare and there is little information about underlying pathogenesis of these diseases in any species. We evaluated CA from formalin-fixed paraffin embedded (FFPE) tissues collected from archival materials of cases of hydrops allantois submitted to University of Kentucky Veterinary Diagnostic Laboratory (n = 10) compared to FFPE of pregnancy-matched normal control mares. RNA was isolated from FFPE tissues from both groups for assessment of expression of genes related to angiogenesis and steroidogenesis.²⁵ Capillary density was reduced and expression of angiogenic genes was lower in CA from hydrops allantois cases, while transcripts related to hypoxia increased compared to controls (Figure 3).²⁶ Interestingly, expression of genes associated with estrogen synthesis and estrogen receptors were also downregulated, which suggests a possible role of estrogen in dysregulation of placental angiogenesis in these cases.

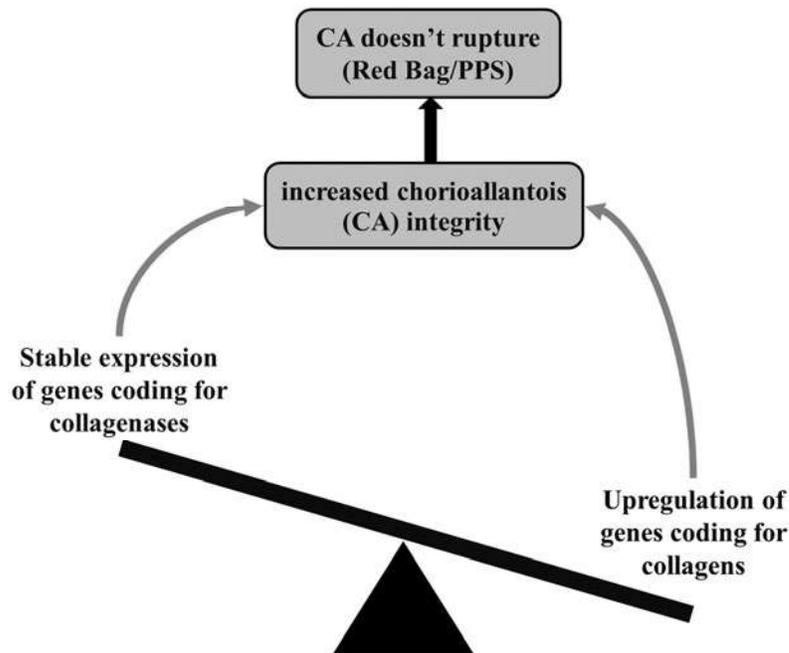


Figure 2. Schematic diagram of changes associated with premature placental separation in mare.

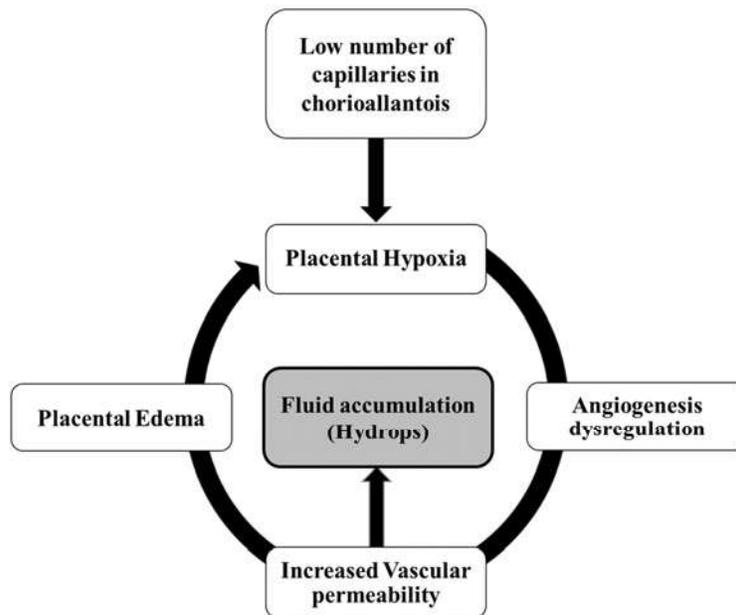


Figure 3. Schematic illustration of changes associated with hydrops allantois in mare.

Proteomic analysis of equine fetal fluids in normal and abnormal pregnancy

Analysis of biological materials by mass spectrometry can also be used to determine protein composition of body fluids, including amniotic and allantoic fluids.^{27,28} Proteome of fetal fluids from control mares and from mares with experimentally induced placentitis was determined by LTQ Orbitrap mass spectrometry.^{27,28} Overall, a total of 130 proteins were characterized in amniotic and/or allantoic

fluid, with a total of 18 proteins upregulated in amniotic fluid from mares with placentitis. Three proteins (haptoglobin, plasminogen isoforms) were present only in amniotic fluid in placentitis. An additional 15 proteins were upregulated in amniotic fluid, including proteins in serpin superfamily, immunoglobulins, apolipoproteins, transferrin, thyroxine binding globulin and serum albumin.²⁸ Interestingly, both, positive acute phase proteins (haptoglobin, alpha-1-antiproteinase, and alpha-2-macroglobulin) and negative acute phase proteins (transferrin albumin) were increased in amniotic fluid from mares with inflamed placenta. A number of these proteins are regulated by inflammatory modulating cytokine (IL6) and change during placental inflammation in women.²⁸ Allantoic fluid had relatively fewer proteins change in placentitis presence and most of these proteins were in common with those of amniotic fluid (alpha-1-antiproteinase, serotransferrin, and transferrin). Similar results were obtained in a second study (increases in transferrin, lactoferrin and alpha-1-antiproteinase in allantoic fluid of mares with placentitis).²⁷ Alpha-1-antiproteinase is an anti-inflammatory protein and modulates tissue-damaging effects of neutrophil enzymatic proteins. Serum concentrations of this protein are used as an acute-phase protein to detect inflammation in humans.²⁷

Conclusion

Application of high-dimensional biology to normal and abnormal equine pregnancy is essential. Data from our studies provide researchers a valuable resource to address specific research questions and to formulate new research hypotheses concerning pregnancy in mare.

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Conflict of interest

None to report.

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Cervix and myometrium: role in preterm and normal term birth in mare

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Abstract

Myometrial activation and cervical remodeling during term and preterm labor involve complex molecular mechanisms. Progress in elucidating these mechanisms has been slow and based on classical molecular techniques such as realtime quantitative reverse transcription polymerase chain reaction. Recently, using ribonucleic acid sequencing, we have elucidated key regulators and molecular mechanisms triggering these events. Objective is to summarize changes in myometrial and cervical transcriptome during placentitis and normal parturition period in comparison to normal pregnancy.

Keywords: Mare, cervix, myometrium, pregnancy, progestins, parturition, placentitis

Introduction

Placentitis induced preterm labor and term labor share a common pathway that includes 3 major events: myometrial activation, cervical remodeling and chorioallantois activation (separation and rupture).¹⁻⁴ Understanding molecular mechanisms of these 3 events holds potential for development of new diagnostic tools and therapies to forestall placentitis induced preterm labor. Recently, using a transcriptomic approach (i.e. ribonucleic acid sequencing), we elucidated key regulators and molecular mechanisms, triggering these events in an experimental model of acute ascending placentitis. Objective is to summarize our recent findings, with a focus on mechanisms underlying myometrial activation and cervical remodeling during equine placentitis and normal parturition period in comparison to normal pregnancy.

Myometrial activation

Key event in placentitis induced preterm labor and term labor is myometrial activation with subsequent initiation of labor.^{1,5} Myometrial activation involves complex myriad of coordinated changes involving immune and hormonal factors, upregulation of several contraction-associated proteins (CAPs), and arrangement of cytoskeletal machinery that provides uterus capacity to generate force and contract.^{1,5} Mechanisms underlying myometrial activation during equine placentitis and normal parturition period are summarized (Figures 1 and 2).

Progestins and progesterone receptor signaling (ligand receptor signaling)

In mammals studied,⁶⁻⁸ progestins, acting through its nuclear receptor (PR, also known as PGR), plays a central role in maintaining myometrial quiescence during pregnancy through blockage of inflammatory cascade and suppression of CAPs. Recently, we reported that myometrial tissue concentrations of 5α dihydroprogesterone (5α DHP, also known as DHP), allopregnanolone (3α DHP) and 20α hydroxy 5α dihydroprogesterone (20α DHP) were lower (progestin withdrawal) in mares with experimentally induced acute placentitis compared to age- and pregnancy-matched controls.¹ This local reduction in 5α DHP and its downstream metabolites is attributed to a decline in enzymes responsible for synthesis of these progestins, such as 5α reductase type 1 (5α R1 also known as SRD5A1) and aldo-keto reductase family 1 member C23 (AKR1C23, also known as AKR1C1).¹ Moreover, expression of nuclear PR is downregulated (functional progestin withdrawal) in equine myometrium during placentitis. A closer look at PR-isoforms proteins (i.e. PR-A and PR-B) revealed a decrease in PR-B to PR-A ratio.¹ It is worth noting that in primates, progesterone (P_4) promotes myometrial quiescence through PR-B-mediated antiinflammatory actions.⁹ At labor, PR-A becomes more predominant and inhibits antiinflammatory actions of PR-B and stimulates proinflammatory gene expression.⁹ Downregulation in progestin-PR signaling is in turn associated with activation of NF- κ B pathway and upregulation of proinflammatory cytokines (e.g. IL1 β), as well as upregulation

of transcripts coding for CAPs (e.g. PTGS2 and GJA4).¹ Together, these findings suggest that placentitis induces localized progesterin withdrawal and progesterin functional withdrawal in myometrium that lead to myometrial activation through activation of inflammatory cascade and upregulation of CAPs.

In prepartum myometrial transcriptome, although we did not identify any significant changes in genes coding for SRD5A1, AKR1C1 and PR, we identified upregulation of aldehyde dehydrogenases (ALDH1A1, ALDH1A2, and ALDH1A3). It is worth noting that ALDH1 family is involved in conversion of retinaldehyde to retinoic acid, which in turn decreases PR transcription.¹⁰ Therefore, upregulation of ALDHs during prepartum period might contribute to myometrial preparation for labor.

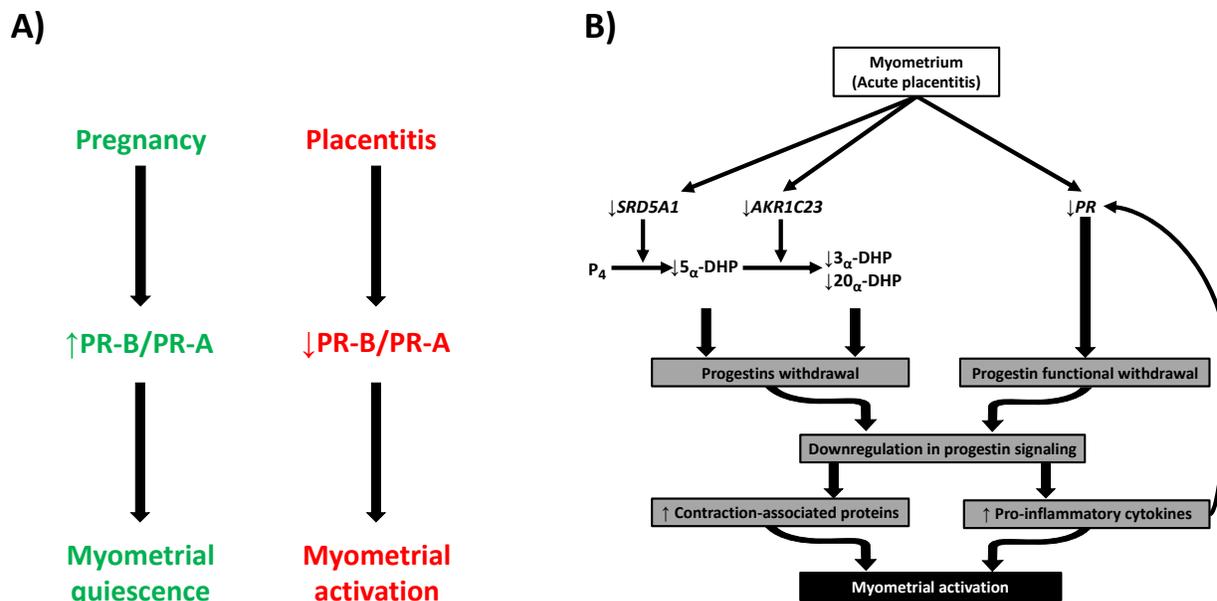


Figure 1. Progesterin-progesterone receptor signaling during equine placentitis. A) PR-B/ PR-A ratio in myometrium during pregnancy and placentitis. B) Molecular mechanisms underlying progesterin withdrawal and progesterin functional withdrawal in equine myometrium during placentitis.

Activation of inflammatory cascade in myometrium

During inflammatory reaction in pathogenic infection, inflammatory cascade is initiated by immune recognition of pathogen mediated through toll-like receptors (TLRs), which are primary and earliest detection mechanisms for pathogens.¹¹ Among known TLRs, TLR2 and TLR4 are responsible for recognition of gram positive and negative bacteria, respectively.¹² Recently, we identified *Streptococcus* induced placentitis to be associated with upregulation of TLR2 in myometrial samples retrieved from placentitis group in comparison to control group.² Moreover, this upregulation is associated with upregulation of a wide array of TLR2-dependent downstream molecules in inflammatory cascade.² These findings highlight central role of TLR2 in triggering inflammatory signals in myometrium during *Streptococcus* induced placentitis. Therefore, targeting TLR2 through therapeutic inhibition (antagonism) might be beneficial for prevention and/or treatment of *Streptococcus* induced placentitis. This notion is supported by reports in primates that treatment of amniotic infection using TLR antagonists (TLRA) resulted in a downregulation of proinflammatory cytokines with subsequent delay or prevention of preterm birth.¹³

In prepartum myometrium, we identified upregulation of several inflammation related genes (e.g.

TNFAIP6, ICAMI, SOCS3, CXCR4).² Upregulation of these genes might reflect presence of sterile inflammatory signaling in myometrium during prepartum stage in mare.

Myometrial infiltration with leukocytes

In women, myometrial infiltration with leukocytes is hallmark of switching myometrium from a quiescent to a contractile state during term and preterm labor.¹⁴⁻¹⁶ Similarly, we reported that equine placentitis is associated with increased myometrial infiltration with leukocytes.^{1,2} Moreover, we elucidated chemokine signaling mechanisms implicated in upregulation of several chemotactic factors including; C-C Motif Chemokine Ligand (CCL2, 4, and 8), C-X-C Motif Chemokine Ligand (CXCL1, 2, 3, 6, 8, and 9) and calgranulins (S100A8 and S100A9).^{1,2} In equine prepartum myometrium (330 days GA), although we identified upregulation of CCL2, CXCL1, CXCL3, and CXCL6, we did not observe marked leukocytic infiltration in myometrium.² This might reflect an early chemotaxis event taking place in prepartum myometrium in preparation for labor.

Myometrial Apoptosis

During placentitis, equine myometrium is associated with a significant upregulation of apoptosis related transcripts, including: caspases (CASP3, CASP4, CASP7), activating transcription factor 3 (ATF3), fas cell surface death receptor (FAS), fos proto-oncogene subunit (FOS), activator protein 1 (AP-1), and baculoviral IAP repeat containing 3 (BIRC3).² Myometrial apoptosis occurred during chorioamnionitis in women^{17,18} and infection-induced labor in mice.¹⁹ Additionally, apoptosis by itself might be a key event in switching myometrial cells from quiescent to contractile status.^{17,18,20}

Uterine contraction associated genes

Placentitis induced myometrial inflammation is associated with upregulation of several contraction-associated transcripts, including prostaglandin endoperoxide synthase 2 (PTGS2, also cyclooxygenase 2; COX2), prostaglandin E Receptor 3 (PTGER3), gap junction alpha 4 (GJA4, also known as connexin-37; CXN37 or CX37), matrix metalloproteinases (MMP1 and MMP8) plus downregulation relaxin (RLN).² Role of these genes in myometrial activation during term and preterm labor is well established in women and mice.²¹ For example, PTGS2 is essential for synthesis of prostaglandin F_{2α} (PGF_{2α}), which is a potent uterotonic (ecbolic).²¹ Contrarily, GJAs are believed to play a critical role in preterm and term labor by forming gap junctions in myometrium, which increase myometrial cell coupling with subsequent generation of synchronous myometrial contractions.²¹⁻²⁴

Cervical remodeling

Cervical remodeling is transformation of cervix from a rigid, tightly closed structure into a flaccid and open one to permit fetal delivery during term and preterm labor.²⁵⁻²⁷ Cervical remodeling consists of 4 overlapping phases (i.e. softening, ripening, dilation, and postpartum repair).²⁵⁻²⁷ Cervical remodeling requires decreases in cervical collagen concentrations (i.e. extracellular matrix (ECM) degradation) and wide dispersing of collagen fibers through increasing cervical water content (i.e. cervical hydration), with subsequent decrease of cervical tensile strength to allow cervical dilation.²⁸ Mechanisms underlying cervical remodeling events during equine placentitis and normal prepartum period are summarized below.

Extracellular matrix (ECM) degradation

During placentitis, we reported upregulation of several proteases, including MMPs (e.g. MMP1, 8, 13 and 14).²⁹ These MMPs are believed implicated in cervical collagen degradation during equine placentitis.²⁹ Contrarily, cervix from prepartum mares (330 d GA) was not associated with significant change in MMPs expression.

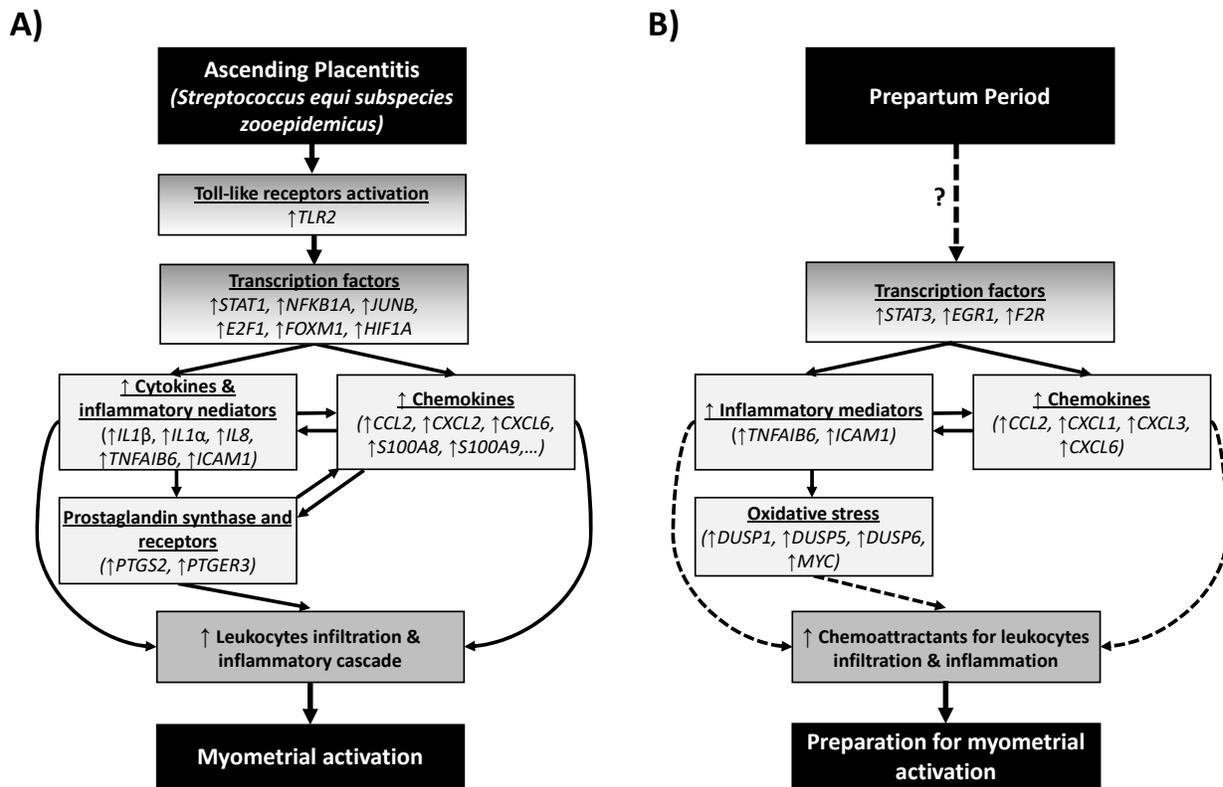


Figure 2. Inflammatory events and associated molecular mechanisms in equine myometrium during placentitis (A) and prepartum period (B).

Cervical hydration

Along with ECM degradation, cervical hydration is another important component in cervical remodeling in which water content increases in cervical tissue, reducing collagen density.²⁹ Cervical hydration could be achieved through various mechanisms, e.g. upregulation of aquaporins (AQPs) water channels and hydrophilic proteoglycans, as well as increased vascular permeability.^{25-27,29} For instance, cervical remodeling during placentitis is associated with upregulation of AQP9 (water transporter), aggrecan (ACAN; a hydrophilic proteoglycan), plus vascular permeability-related genes such as Rac family small GTPase (RAC) and nitric oxide synthase (eNOS, also known as NOS3).²⁹ Altogether, these findings highlight possible molecules implicated in cervical ECM degradation and cervical hydration during placentitis.

Conclusion

This review provides a brief overview of key regulators and molecular mechanisms underlying myometrial activation and cervical remodeling during placentitis and prepartum period. Strategies to block identified key regulators and associated pathways (e.g. using TLRAs) hold potential for therapies to forestall placentitis-induced preterm birth.

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Conflict of interest

None to report.

Abbreviations

20 α DHP; 20 α -hydroxy-5 α dihydroprogesterone
3 α DHP; allopregnanolone
5 α -DHP; 5 α -dihydroprogesterone
AKR1C23; aldo-keto reductase family 1 member C23
ALDH1; aldehyde dehydrogenases
AP-1; Activator protein 1
AQPs; aquaporins
ATF3; activating transcription factor 3
BIRC3; baculoviral IAP repeat containing 3
CAPs; contractions-associated proteins
CASP; caspase
CCL; C-C Motif Chemokine Ligand
CXCL; C-X-C Motif Chemokine Ligand
CXCR4; C-X-C motif chemokine receptor 4
DUSP; Dual Specificity Phosphatase
ECM; Extracellular matrix
eNOS/NOS3; nitric oxide synthase
FAS; Fas Cell Surface Death Receptor
GJA4/CXN37; gap junction alpha 4/connexin-37
ICAM1; intercellular adhesion molecule 1
IL; Interleukin
MMP; matrix metalloproteinases
MYC; MYC Proto-Oncogene, BHLH Transcription Factor
NF- κ B; Nuclear factor- κ B
P4; progesterone
PGF_{2 α} ; Prostaglandin F_{2 α}
PR/PGR; Progesterone receptor
PTGER3; Prostaglandin E Receptor 3
PTGS2/COX2; prostaglandin-endoperoxide synthase 2/Cyclooxygenase 2
RAC; Rac family small GTPase
RLN; relaxin
S100A; calgranulins
SRD5A1; 5 α reductase type 1
TLR; Toll-like receptors
TLRA; TLR antagonists
TLRs; toll-like receptors
TNFAIP6; TNF alpha induced protein 6

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Management of dystocia in the mare
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Abstract

A dystocia is an emergency condition in the mare. The goals of dystocia management are to save the life of the foal, save the life of the mare, and to preserve future fertility of the mare. Early detection and rapid appropriate intervention are critical for foal survival. The most common cause of an equine dystocia is an abnormality of fetal posture.

Keywords: Dystocia, foaling, mare, obstetrics

Introduction

The term dystocia refers to an abnormal or difficult birth.¹ Dystocia or difficult birth occurs in approximately 4% of Thoroughbred foalings.² Abnormal orientation of the fetus in the birth canal (i.e. postural abnormalities) is the most common cause of dystocia in the horse. Dystocia is more common in mares during their first foaling (i.e. maiden mares) than in mares that have previously given birth to 1 or more foals. The ultimate goals in assisting in an equine dystocia are to save the life of the foal, save the life of the mare and to preserve the future fertility of the mare.

Time management during a dystocia

Active labor (Stage II) is a rapid event in most mares, with most foals delivered within 20 - 30 minutes after rupture of the chorioallantoic membrane.³ The average length of Stage II for mares in 1 study was 16.7 minutes, with 71.7% of foals born in less than 20 minutes.⁴ An owner or foaling attendant should be concerned if a foal has not been born or if significant progress toward birth has not occurred within 20 minutes after rupture of the outer chorioallantoic membrane and subsequent release of allantoic fluid (i.e. when a mare 'breaks her water').

Dystocia and foal survival

Dystocia in mares is a medical emergency and time is critical.⁴⁻⁶ It has been estimated that each 10 minutes increase in the duration of Stage II labor beyond 30 minutes is associated with a 10% increase in the existing risk of a foal being born dead and a 16% increase in risk of the foal not surviving to discharge from a referral hospital or clinic.⁵ An additional study reported that the interval from rupture of the chorioallantoic membrane to delivery of the fetus was 13.6 minutes shorter for foals that were born alive and survived to discharge than for foals born dead or that did not survive to discharge.⁷ A third study noted a significant increase in stillbirth, foal morbidity and foal mortality occurred when the duration of Stage II of labor was greater than 40 minutes (Table 1).⁴ Combined, these statistics reinforce the concept that early accurate detection of a foaling problem and early effective intervention is critical to foal health and survival.

Early detection of an equine dystocia and rapid appropriate intervention in problem cases are critical for foal survival. Placental separation and subsequent decrease in oxygen supply in utero are likely the primary factors that lead to fetal death during a prolonged dystocia. If the fetus is still within the uterus (i.e. not fully engaged into the birth canal) and the placenta is still attached and the umbilical cord is not compressed or disrupted, the oxygen supply to the fetus may be sufficient for short-term survival. However, it is critical that the fetus be delivered as safely and expeditiously as possible.

Dystocia management plan

It is strongly recommended that an emergency plan be formulated prior to the foaling season to prepare for a difficult birth, a medical problem with a newborn foal, or medical issues with the mare. The plan should be the result of a conversation among the mare owner, breeding farm/ranch manager,

Table 1. Stillbirth, foal morbidity, and foal mortality associated with duration of Stage II of labor

Duration Stage II (minutes)	(n)	Stillbirth (%)	Morbidity (%)	Mortality (%)
0 - 10	173	1.7 ^a	4.6 ^a	4.1 ^a
11 - 20	549	0.4 ^a	3.3 ^a	2.2 ^a
21 - 30	211	2.8 ^a	6.6 ^a	5.2 ^a
31 - 40	45	0 ^a	2.2 ^a	4.4 ^a
41 - 50	13	7.7 ^b	7.7 ^b	7.7 ^b
> 50	18	22.2 ^b	11.1 ^b	27.8 ^b

^{a,b}Within a column, percentages without a common superscript differed ($p < 0.05$)

foaling attendant and the veterinarian(s). All personnel actively involved in foaling mares should be trained and ready to assist. A foaling kit should be readily available near the foaling stall. Management of a dystocia is dependent on training, experience and availability of farm personnel, as well as the proximity of veterinary services.

Early communication in the event of a dystocia

It is recommended that a foaling attendant call for assistance (on-farm personnel or veterinary assistance, as appropriate) in the following circumstances:

- If there has been no progress toward delivery after 15 - 20 minutes after rupture of the chorioallantois
- If progress toward delivery abruptly stops
- If the mare becomes acutely painful or exhibits signs of shock
- If the attendant is confident that a problem with foaling exists
- If the attendant is unsure if a problem with foaling exists
- If the attendant does not have the knowledge, training, or ability to identify or correct the problem

Causes of dystocia in the mare

The most common causes of dystocia in the mare categorized by difficulty of management or correction are listed (Table 2). Mild dystocias are commonly managed on the farm by foaling personnel. More complicated dystocias may require advanced training or experience and often require veterinary intervention. Veterinary advice or assistance should immediately be sought if the problem cannot be safely and quickly corrected by on-farm/ranch personnel.

Fetal alignment in the birth canal during foaling is described in obstetrical terms as:

- Presentation - relationship of the long axis of the fetus to the dam's birth canal
- Position - relationship of the dorsum of the fetus to the quadrants of the maternal pelvis
- Posture - relationship of the fetal extremities to the fetus itself

Alignment of the fetus in the birth canal during a normal delivery is described as:

- Anterior presentation, dorsal-sacral position, with both forelimbs extended and the head extended

Failure of the fetus to be properly oriented in the birth canal as foaling progresses will almost always prevent normal passage and result in dystocia. The most common causes of dystocia in the mare are abnormalities of fetal posture (i.e. abnormal alignment of the head or forelimbs).⁸ A retrospective study of over 1,000 equine births noted that abnormalities of fetal posture were associated with 37.7% of dystocias, including 1 or both front limbs retained (30.1%), retained fetal head (3.8%) or both limb(s) and

head retained (3.8%).⁴ A 0.8% incidence rate of ‘hip-lock’ (feto-pelvic disproportion) was recorded. Premature placental separation was recorded in 1.6% of foalings. A periparturient hemorrhage event was noted in 0.8% of foalings.

Table 2. Common causes of dystocia in mares, arranged by degree of difficulty in correction

Correction difficulty	Foaling complication or issue
Mild	Elbow lock Upside-down foal Backwards foal Uterine inertia ‘Red-bag’ (Premature placental separation)
Moderate	Front leg(s) flexed at the knee (carpus) Neck flexed ventrally; muzzle below pelvic brim Feto-pelvic disproportion or ‘Hip-lock’
Difficult	Front leg(s) flexed at shoulder Neck flexed to side; muzzle not reachable Backwards presentation, hind leg(s) flexed at hip Transverse presentation Twins (when both entering birth canal simultaneously)

Initial physical examination of the mare during a dystocia

A brief physical examination should be performed to evaluate the health status of the mare.⁹ It is recommended that the examination be performed in a large stall or other open area or in stocks that can quickly and completely be taken apart with removal of a few pins. It is not recommended to examine a pregnant mare experiencing a dystocia in solid-sided stocks, because many foaling mares will attempt to lie down during vaginal examination or fetal manipulation. In addition, young inexperienced foaling mares may be exceptionally nervous, excited or scared during their first delivery and these behaviors may be even more pronounced during a difficult birth.

Safety of personnel should be of paramount concern during all examinations and obstetrical procedures. Consequently, it may be necessary to sedate the mare, apply a twitch or perform an epidural to facilitate examination, provide pain relief and/or decrease straining.

Intervention in an equine dystocia

Choices for relieving an equine dystocia include an assisted or controlled vaginal delivery, cesarean surgery (cesarean section) or fetotomy (Figure 1). The final decision may depend on the status of the fetus, duration and severity of the dystocia, economic value of the mare and fetus, clinician expertise, client preference, facilities available and other considerations. Intervention by trained personnel may save the life of a foal in the event of a dystocia. However, the undisputed key factors in foal survival are early recognition of a foaling difficulty by breeding farm personnel and an early call for assistance.

Assisted vaginal delivery

A reproductive examination is performed to identify the obstetrical problem, determine if the fetus is alive and to formulate a plan. If possible, the tail of the mare is quickly wrapped or placed in a plastic sleeve and held out of the way. A brief wash and rinse of the perineal area is performed to remove debris and manure. A manual vaginal examination is subsequently performed, with or without application of a sterile obstetrical sleeve. The goals are to determine:

- If the chorioallantoic membrane had indeed ruptured (i.e. did the mare really ‘break her water’). In some cases, the mare may not actually be in Stage II of labor or the signs of abdominal pain may be colic in nature and not associated with uterine contractions.

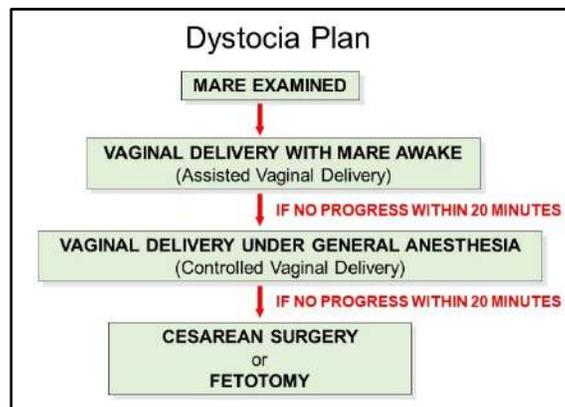


Figure 1. General plan for dystocia management in a mare

- Dilation status of the cervix
- Presentation, position and posture of the fetus (i.e. orientation of the fetus in the birth canal)
- Live/dead status of the fetus (if possible). Accurate assessment of fetal viability may be critical to subsequent obstetrical decisions. In some cases, fetal viability status cannot be determined and one should proceed assuming that the fetus is viable. Evaluation for fetal viability may include:
 - Reflex reactions upon application of pressure to the tongue or the eye
 - Detection of a suckle response after insertion of finger into mouth
 - Detection of a heart-beat in the chest (if reachable)
 - Detection of a pulse within the umbilical cord (if reachable)
 - Detection of fetal movements, etc.
- If perineal, vaginal, cervical or other problems have already occurred

If only legs or feet are presented at the vulva, one needs to determine if they are front legs or hind legs, as subsequent management decisions are dramatically different. The best way to differentiate front legs from hind legs is to assess the direction of flexion of the 2 largest moveable joints above the foot.

- In front legs, the 2 joints (fetlock and carpus) flex in the same direction
- In hind legs, the 2 joints (fetlock and hock) flex in opposite directions.

A plan for intervention (modified from a predetermined dystocia plan) should be developed for each specific dystocia, based on the information above, the health status of the mare, duration of dystocia, economic value of the mare and foal and other factors. In addition, a backup plan should always be discussed in the event that the initial plan is not successful.

Normal fetal orientation in birth canal

A vaginal delivery may be attempted with the mare awake and either standing or recumbent if the fetus is lined up or oriented normally in the birth canal (anterior presentation, dorsal-sacral position, with both forelimbs and the head extended) or in posterior presentation with both hind limbs extended. A normal fetal orientation may be present in cases of uterine inertia or in cases of fetal oversize relative to the birth canal of the mare (feto-pelvic disproportion; ‘hip-lock’). The following guidelines are recommended when assisting delivery:

- If the mare is straining and will not allow a vaginal examination, consider sedation or administration of an epidural
 - Options for an epidural using an 18 gauge, 3 inch needle include:
 - Lidocaine (2%) 5 - 8 ml
 - Xylazine (0.17 mg/kg) diluted in 10 ml of 0.9% sterile saline
 - Combination of lidocaine (0.22 mg/kg), plus xylazine (0.17 mg/kg)
 - Pump obstetrical lubricant into the uterus around the fetus. The commercial product ‘J-Lube’ (Jorgensen Laboratories, Loveland, CO) is an excellent obstetrical lubricant,

but should be used with caution if a uterine tear is present or suspected or if a cesarean surgery is being considered. J-Lube has been reported to cause severe peritonitis if leaked into the abdominal cavity.¹⁰

- Repel the fetus back into the abdominal cavity (if necessary) to correct any abnormalities of presentation, position or posture
- Confirm that the fetus is correctly lined up in the birth canal
- Place obstetrical chains or foaling straps on each front leg above the fetlocks
 - Obstetrical chains are advantageous in that they are easy to place, a hook or handle can be attached at any location on the chain and the chain and hooks can be easily cleaned and either cold sterilized or autoclaved
 - Wide-webbed nylon obstetrical straps can be advantageous in that a loop can be made on each end and the strap itself becomes the handle; the disadvantage is that they are not as easy to clean or sterilize as chains
 - Thin rope, baler twine or other narrow devices should not be used on foals due to the potential for damage to skin, tendons or other structures
- Pull when the mare has a uterine contraction; stop pulling when the mare relaxes in-between contractions
- Do not exceed the force of 2 people when assisting
- Avoid use mechanical devices to pull a foal
- Pull in an outward (initially) and then slightly downward direction
- ‘Walk’ the shoulders of the foal through the birth canal, 1 at a time

Uterine inertia

In some cases, a mare may not provide sufficient uterine contractions (or any apparent uterine contractions) to deliver a fetus, despite the fact that the fetus is aligned properly in the birth canal. In other cases, the mare may become exhausted and unable to muster additional uterine contractions. In these instances, the following guidelines should be followed:

- Confirm that the foal is in correct presentation, position and posture
- Apply obstetrical lubricant around the fetus (if needed)
- Attach obstetrical chains or straps
- Provide traction to deliver the fetus

Abnormal fetal presentation, position or posture

If the fetus is not in a normal orientation in the birth canal, the abnormality must be corrected prior to any attempt to apply traction (i.e. do not pull on the fetus). It is imperative to accurately assess the abnormality so that proper and expedient intervention can be initiated. The most common disorders of presentation, position or posture in an equine dystocia include:

- Elbow lock
- Front leg(s) flexed at the knee or shoulder
- Head flexed laterally or (less commonly) flexed ventrally
- Posterior presentation

Elbow lock

Elbow lock is a common and relatively mild abnormality requiring minimal intervention by the foaling attendant. The foal will be in anterior presentation, usually a dorsal-sacral position, with both front feet and a nose or the cranial portion of the head visible. However, 1 front leg is clearly protruding further than the other, which may be visible only to the fetlock. The nose of the foal is positioned near the mid-cannon bone area of the most advanced front leg. Only 1 leg and the head advances with each uterine contraction; the second front leg does not advance because the elbow is ‘locked against’ or ‘caught on’ the pelvic brim. When ‘elbow lock’ is recognized, the foaling attendant should immediately intervene. Traction is applied to the retained limb when the mare relaxes between uterine contractions. A ‘pop’ is

often felt when the retained elbow is freed from the pelvis. The foal is usually delivered without further assistance with subsequent contractions.

Posterior presentation

Foals in a posterior presentation may be impeded from a smooth delivery if the tail-head becomes wedged on the dorsal pelvis of the mare. In addition, there is a possibility that the umbilical cord may be compressed on the ventral pelvis of the mare, potentially reducing blood flow and decreasing oxygen transport to the fetus. In contrast to an anterior presented foal, there is no possibility that a foal in a posterior presentation could breathe on its own if progress halts during delivery. For all of the above reasons, it is generally recommended that a posterior presented fetus be delivered as quickly and efficiently as possible, with assistance provided as needed.

Feto-pelvic disproportion

Feto-pelvic disproportion or 'hip-lock' is much less common in horses than cattle. However, it is a significant event when it does occur. Normally the 'test for delivery' in a foal in anterior presentation is passage of the second shoulder through the pelvis. In a majority of equine deliveries, if the shoulders fit through, the hips will pass uneventfully. Unfortunately, a mild to severe form of 'hip-lock' occurs in ~ 0.5 - 1.0% of foalings.⁷ A recommended strategy for cases of 'hip-lock' is to infuse a generous volume of obstetrical lubricant around the fetus and into the uterus followed by rotation of the fetus ~ 30 - 45° to take advantage of a slightly wider internal pelvic diameter at that position and then apply traction. General anesthesia may be necessary if the mare becomes intolerant of the procedures.

Developmental abnormalities such as contracted tendons, wry neck or wry nose, a twisted neck or other conditions may prevent or at least delay correction of fetal posture.

Controlled vaginal delivery

If significant progress is not made within 15 - 20 minutes with the mare awake, the mare may be placed under general anesthesia and a controlled vaginal delivery attempted. General anesthesia is used to facilitate safe evaluation of the mare and to reposition the fetus into the correct orientation to allow for a controlled vaginal delivery. Induction of anesthesia is advantageous to eliminate uterine contractions and straining by the mare. However, lack of contractions means that the fetus must be delivered by traction applied from the outside.

In the field, general anesthesia can be accomplished by premedication with xylazine (1.1 mg/kg, IV) with or without addition of butorphanol tartrate (0.01 mg/kg, IV), followed by induction with ketamine (2.2 mg/kg, IV), with or without addition of diazepam or midazolam (0.05 - 0.1 mg/kg, IV). Additional doses of xylazine/ketamine may be administered as needed to maintain general anesthesia while the dystocia is being resolved.

Elevation of the hindquarters of an anesthetized mare may be beneficial to increase space in the caudal abdominal cavity and therefore make it easier to reposition fetal body parts that are not aligned properly in the birth canal. Liberal application of obstetrical lubricants along all sides of the fetus and within the uterine cavity will greatly enhance repositioning and subsequent extraction of the fetus.

In summary, a controlled vaginal delivery with the mare under general anesthesia, if successful, obviates the need for more invasive and expensive procedures such as a cesarean surgery or a fetotomy. The mare is allowed to recover and is reunited with the foal (if alive) when appropriate.

Cesarean surgery

If significant progress toward an assisted or controlled vaginal delivery is not forthcoming, a cesarean surgery or other procedures may be indicated. In the horse, a cesarean surgery is almost exclusively performed in a designated operating room at a veterinary hospital or other referral center.^{11,12} Unlike the situation in cows, cesareans are almost never performed in the field or other open areas. A brief description of the cesarean surgery is as follows:

- The mare is anesthetized and placed in dorsal recumbency

- The ventral abdomen is clipped, scrubbed and draped in preparation for surgery
- An incision is made along the ventral midline through which the uterus is subsequently exteriorized
- An incision is made into the uterus and the foal is delivered and attended to by medical staff
- Hemorrhage from the uterine incision is controlled using a synthetic absorbable suture in a simple continuous pattern followed by an inverting Cushing pattern
- The linea alba is closed in a simple continuous pattern; the subcutaneous tissue is closed using synthetic absorbable suture; the skin is subsequently closed with sutures or surgical staples
- The mare is subsequently moved to a recovery stall

Terminal cesarean surgery

In exceedingly rare emergency circumstances, a pregnant mare may be placed under general anesthesia, a cesarean surgery performed and the foal delivered, and the mare subsequently euthanized while still under anesthesia. Situations may include severe musculoskeletal issues, ruptured prepubic tendon or other conditions. It is recommended that the breeding history and prospective due date be reviewed and milk samples be collected and calcium concentration measured to evaluate fetal maturation. If time permits, dexamethasone (i.e. 100 mg, as an intramuscular injection for a 450 kg mare) may be administered once daily for up to 3 days to promote fetal maturation prior to the cesarean surgery.

Fetotomy

The term fetotomy refers to the surgical dissection of a dead fetus in utero.^{13,14} The procedure is performed in the event of a dystocia that cannot be resolved by standard assisted or controlled vaginal delivery techniques and when a cesarean surgery is not an option. The goal is to save the life of the mare and protect her future reproductive potential. Specialized obstetrical instruments are used to perform a fetotomy, the most common of which is a fetotome and obstetrical wire. The wire is passed around a retained fetal body part and is used to dissociate that part from the rest of the fetus to allow for delivery of the fetus. Examples of when a fetotomy might be performed on a dead fetus include:

- Breech presentation in which a fetus is in posterior presentation with both hind legs flexed at the hip
- Flexed neck that cannot be extended into a normal position
- Front leg(s) retained/flexed at the shoulder and cannot be extended

Foal care following a dystocia

Resuscitation equipment should be available on farms that foal out a lot of mares and farm personnel should be trained in the proper care and use of the equipment. A resuscitation bag (i.e. Ambu bag) attached to a face mask is a simple-to-use device that can be safely applied to foals by farm personnel to help stimulate breathing in a newborn foal. Alternatively, a device called the 'Foal Resuscitator' may be used. Special immediate attention must be given to the foal following a dystocia. The foal is at high risk of many neonatal diseases including neonatal maladjustment syndrome, failure of passive transfer, ruptured bladder and trauma, e.g. rib fractures.

Mare care following a dystocia

The mare should also be examined in due course following resolution of a dystocia. It is common for the placenta to be retained following a dystocia and preventative treatments are often instituted after a dystocia has been relieved. Finally, the reproductive tract of the mare (i.e. perineum, vagina, cervix and uterus) should be carefully examined for trauma that may lead to more severe medical issues and/or limit her future reproductive performance.

Conclusion

Dystocia is a medical emergency and time is critical to optimize foal survival. The key factors in foal survival are early recognition of a foaling difficulty by breeding farm personnel and early appropriate intervention.

Conflict of interest

There are no conflicts of interest to declare.

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Identifying risk factors for mastitis in postpartum bitches

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Mastitis in postpartum bitch is a disease that affects wellbeing of both dam and pups that rely on their mother's milk as the sole source of nutrition during a critical growth period. Risk factors for canine mastitis have not been well-studied and this information could help veterinarians and breeders identify females that may need closer monitoring. Goal of this study was to identify risk factors for postpartum mastitis in bitch. We hypothesized that age, breed, litter size, and whelping interventions are associated with risk of developing mastitis in postpartum bitch. A retrospective cohort study was performed using data from 2 guide dog colonies over a 13-year period. A total of 2,489 whelpings occurred during study period. All bitches were monitored closely and mastitis was diagnosed by colony veterinarian. Risk factors examined were: colony, breed of dam, litter size, neonatal pup loss, age (in years), parity, dystocia, use of oxytocin during whelping, use of calcium during whelping, and if cesarean section was performed. Risk factors were evaluated individually and offered to the model if $p < 0.20$. A final model was built using a backwards-stepwise method and colony was forced into the model to account for colony-specific differences. Logistic regression ANOVA was performed using JMP Pro v. 14 (SAS Institute). Mastitis incidence was 8.9% (222 cases). Three significant risk factors were identified and offered to the model built; all were retained in the final model. First, mastitis risk increased as litter size increased and ROC analysis identified ≥ 9 pups as the optimal threshold. Bitches that had ≥ 9 pups (120/1,048 or 11.5% versus 7.1% in litters < 9 pups) were more likely to develop mastitis (OR = 1.67, 95% CI 1.26 - 2.21; $p = 0.0003$). Second, there was an effect of dam breed; Golden Retrievers (12.9% OR = 2.81, 95% C.I. 1.32 - 5.99; $p = 0.0075$) and Labrador Retrievers (8.6% OR = 2.05, 95% C.I. 1.03 - 4.11; $p = 0.0418$) were more likely to develop mastitis compared to German Shepherd Dogs (4.8%). Finally, bitches that whelped naturally (9.4%) were more likely to develop mastitis compared to bitches that had cesarean section (5.7%) performed (OR = 1.70, 95% CI 1.04 - 2.76; $p = 0.0332$); therefore, bitches that underwent cesarean section had lower rates of mastitis. Surprisingly, bitches with singletons (3%) had lower mastitis risk than bitches with large litters, and neonatal (> 3 days) loss of pups did not affect mastitis risk. The dogma of older bitches having higher risk of mastitis was also not observed in our population. There is an effect of dam breed on mastitis risk and further studies examining more breeds are warranted.

Keywords: Canine, mastitis, risk factors

Fungal growth is more likely to be affected by hormones in equine uterine isolates compared to isolates from nonreproductive sites

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Fungal endometritis is an important cause of infertility in mares. Studies indicated that whereas estradiol may have a stimulatory effect on fungal growth, progesterone may have an inhibitory effect. However, both studies only examined 1 fungal isolate. Hormone responsiveness of fungal isolates from mare reproductive tract has not been evaluated. Objective was to determine if clinical fungal isolates from mare uteruses are hormone responsive; if so, are uterine isolates more likely to be hormone responsive compared to fungal isolates from nonreproductive sites. We hypothesized that estradiol has stimulatory effects whereas progesterone has inhibitory effects on fungal growth and that fungal isolates from mare uteruses are more likely hormone responsive compared to fungal isolates from nonreproductive sites. Fungal isolates from mare uteruses (n = 7) and isolates from nonreproductive sites (n = 5) were evaluated for hormone responsiveness after being cultured in RPMI media for 48 hours at 30°C in 96-well plates. Absorbance was read at 600 nm at seeding times, 24 and 48 hours. Correlation between absorbance and fungal concentration was determined by hemocytometer. Dependent variable was percent change in fungal concentration from initial seeding. Estradiol treatment groups were: ethanol (vehicle) control and 10, 150, and 1,000 ng/ml whereas progesterone treatment groups were: ethanol control and 0.1, 5, and 100 µg/ml. Data were not normally distributed and nonparametric analyses were used. Effects of hormone treatment and site on fungal growth were determined by Kruskal-Wallis test and treatment effects were determined using multiple comparison rank sum Steel test with ethanol group as the control in JMP Pro v. 14 (SAS Institute, Cary, NC). Estradiol affected growth (p = 0.0004) at 48 hours with increased growth (p = 0.0189) in 10 ng/ml compared to control. Progesterone affected growth at 24 (p < 0.0001) and 48 hours (p < 0.0001) with decreased growth in 5 µg/ml (p < 0.0001) and 100 µg/ml (p < 0.0001) groups compared to controls. Estradiol affected growth (p < 0.0001), with 3/7 (at 24 and 48 hours) isolates from uterus and 0/5 (at 24 hour) and 1/5 (at 48 hour) isolates from nonreproductive sites affected by estradiol. Progesterone affected growth (p < 0.0001), with 6/7 (at 24 and 48 hour) isolates from mare uterus and 1/5 (24 hour) and 3/5 (48 hour) isolates from nonreproductive sites affected. Majority of fungal isolates from mare uteruses were hormone responsive to estradiol (that increased growth rates) and progesterone (that decreased growth rates) whereas most nonreproductive isolates were not hormone responsive. This study highlighted potential interactions between hormone status and fungal infectivity in equine uterus.

Keywords: Fungal, hormone responsiveness, horses

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A novel method to reduce egg laying in companion avian species using a hen model

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Reproductive-related diseases in female avian species are a common problem in zoological companion animal medicine. Of particular concern is a potentially serious condition called egg-binding where eggs are retained within female reproductive tract. In chronic cases, treatment involves preventing egg production with surgical removal of the ovary or administration of hormones such as leuprolide acetate, or deslorelin acetate. These approaches are problematic, as surgical gonadectomies in birds are difficult, if not impossible, and hormonal injections and implants are expensive, variably effective, and short-acting; therefore, an effective nonsurgical method of suppressing egg production is needed. We are developing a nonsurgical approach to reduce gonadal activity and germ cell production using an antiMullerian hormone receptor 2 antibody (AMHR2)-guided lipid nanocomplex carrying a cytotoxin (saporin) to induce apoptosis in gonadal support cells exhibiting AMH2 receptor. This method disrupted gonadal architecture and germ cell development in rats and pigs. We hypothesized that this unique technology would reduce hens egg production. Rhode Island Red laying hens (n = 12) formed control group (n = 6, 0.5 ml sterile intravenous saline) and treatment group ([n = 6], administered 0.5 ml of sterile intravenous saline containing 100 nmol of nanocomplex). Hens were housed separately, weighed twice weekly and photographed once a week. General health and number of eggs laid were evaluated daily starting 13 days prior to treatment until 28 days after treatment. Blood was collected prior to treatment and at end of study for concentrations of estradiol, progesterone and androstenedione. Hens were euthanized via intravenous pentobarbital and left ovary was collected, measured, weighed, and formalin-fixed for H&E histology. There were no differences between groups in general health, body weight, ovarian weight, or blood hormone concentrations. Histologically, all ovaries had evidence of follicular activity. Most obvious effect was cessation of egg laying in all treated hens starting 3 days postinjection and lasting at least 16 days in 4 of 6 treated hens. All injected hens eventually resumed laying eggs.

Eggs collected		Preinjection	Days 0-2	Days 3-18	Days 19-28
		(13 Days)	(3 Days)	(16 Days)	(10 Days)
Controls	Total eggs	59	12	52	31
	Eggs/bird/day	0.76	0.67	0.54	0.52
Nano injected	Total eggs	57	12	10	28
	Eggs/bird/day	0.73	0.67	0.1	0.47

Administration of nanocomplex resulted in short-term cessation of egg laying in all treated hens without impacting overall health of these birds supported the hypothesis. Further studies are needed to determine if a different dose and/or route of administration could increase the length of this cessation, or even lead to a permanent loss of egg production.

Keywords: Gonadal suppression, fertility, avian egg laying, egg-binding, nanocomplex

Sperm-bound antisperm antibodies are associated with poor cryosurvival of stallion sperm

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Semen freezing is a common practice in equine reproduction. However, ~ 30 - 40% stallions' semen does not survive cryopreservation. Reason for poor cryosurvival is not clearly understood. Antisperm antibodies (ASAs) are associated with poor cooling ability of stallion sperm. We hypothesized that presence of sperm-bound ASAs is associated with poor cryosurvival. Objective of this study was to evaluate ASA binding in stallions with semen with good versus poor cryosurvival. Ejaculates from stallions (n = 21) were extended in INRA96 to 40×10^6 sperm/ml and shipped overnight to laboratory in a passive cooling device (Equitainer[®]). After arrival, each ejaculate was divided into 3 aliquots, centrifuged, resuspended in Botucio[®] (BC; Botupharma, Scottsdale, AZ), EZ Mixin Cryomax Modified French[™] (MFR; ARS, Chino, CA) or EZ Mixin Cryomax Lactose-EDTA[™] (LE; ARS) and frozen following manufacturer's instructions. Semen was stored in liquid nitrogen until evaluation. One straw from each aliquot was thawed at 38°C for 30 seconds and was assessed for total and progressive sperm motility (CASA). In addition, acrosomal integrity (FITC-PNA/PI), percentage of apoptotic and necrotic sperm (Annexin V/PI), and percentage of sperm with IgG and IgA binding (antiequine IgG and IgA) were evaluated with flow cytometry. Postthaw motility was considered acceptable if PM \geq 30%. Semen considered as good cryosurvival if they had acceptable postthaw motility with at least 2 extenders. There was no difference in ASA binding among semen extenders and data were pooled. Semen with good cryosurvival (n = 13) had a lower percentage of IgG ($4.1 \pm 0.5\%$) and IgA bound sperm ($2.9 \pm 0.3\%$) compared to semen with poor cryosurvival (n = 8; IgG $13.5 \pm 2.3\%$, IgA $11.2 \pm 1.7\%$) (p < 0.0001; Student's t-test). None of the semen with good cryosurvival were ASA positive. However, 43.5% of semen with poor cryosurvival were ASA positive (p < 0.0001, Chi Square). There was a negative correlation between percentage of IgG and IgA bound sperm, and total motility (IgG: p = 0.0045, $R^2 = -0.36$; IgA: p = 0.0003, $R^2 = -0.45$) and progressive motility (IgG: p = 0.0005, $R^2 = -0.44$; IgA: p < 0.0001, $R^2 = -0.49$), and a positive correlation between percentage of IgG bound sperm and percentage of sperm with damaged acrosomes (IgG: p = 0.049, $R^2 = -0.36$). In summary, presence of ASAs was associated with poor cryosurvival of stallion sperm.

Keywords: Stallion, semen, antisperm antibodies, cryopreservation, freezing

Effect of energy substrates on cool-stored stallion sperm

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Commercially available equine semen extenders for cooled storage are typically formulated with glucose as an energy substrate. When incubated at 38.5°C, stallion sperm are reported to preferentially utilize oxidative phosphorylation (OXPHOS) substrates (lactate or pyruvate) for energy production. This study was conducted to determine the effect of 3 energy substrates (glucose, lactate, or pyruvate), individually or in combination, on sperm quality after cooled storage for 24 hours. We hypothesized that inclusion of OXPHOS energy substrates in extender would increase sperm quality parameters compared to glucose alone. Three ejaculates from each 1 of 6 stallions (n = 18) were processed by cushioned centrifugation using a milk-based extender containing various energy substrates, or their combinations, and 10% (v/v) homologous seminal plasma. Treatment groups were: no substrate added (Control); 40 mM added glucose (Glu-40); 2 mM pyruvate (Pyr-2); 2 mM lactate (Lac-2); 19.8 mM pyruvate (Pyr-19); 19.8 mM lactate (Lac-19); 40 mM glucose + 2 mM pyruvate + 2 mM lactate (Glu-Pyr-Lac-2); or 40 mM glucose + 19.8 mM lactate + 19.8 mM pyruvate (Glu-Pyr-Lac-19). Extended semen was stored at 2 temperatures, 10 or 20°C, for 24 hours. After storage, semen was analyzed for % total motility (TMOT) using CASA; and % viable/acrosome intact (VAI), and % viable/lipid peroxidation positive (VLPP) using flow cytometry. Data were rank-transformed prior to analysis using General Linear Model procedure. Statistical significance was set at $p < 0.05$. At both storage temperatures, %TMOT was similar ($p > 0.05$) among Glu-40, Glu-Pyr-Lac-2 and Glu-Pyr-Lac-19 (57, 58, 54%), and higher ($p < 0.05$) in these than other treatment groups (31 - 39%; $p < 0.05$). Percent VAI was lower ($p < 0.05$) in Control (68%) and Pyr-2 (69%), than in Glu-40 (73%). Percent VLPP was similar ($p > 0.05$) among Glu-40, Glu-Pyr-Lac-2 and Pyr-19 (22, 24, 22%, respectively), but higher ($p < 0.05$) in these groups as compared to the Control (17%). These results suggest that at reduced storage temperatures, addition of OXPHOS substrates in extender do not enhance sperm motility over that of glucose alone. Only slight differences on %VAI were observed, which might suggest that type of energy substrate does not have a substantial impact on stallion sperm membrane intactness. Higher % VLPP was observed in treatment groups with higher motility values, which may be related to a higher metabolic rate, and resultant production of reactive oxygen species.

Keywords: Stallion, sperm, low temperature, energy substrate, motility, plasma membrane

Effects of recent feeding on canine serum progesterone

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Serum progesterone values are commonly used to estimate luteinizing hormone (LH) surge in the bitch to appropriately time insemination. Known factors affecting serum progesterone values include type of sample tube used, size of dog, age of dog, anticoagulant, interval from blood collection to centrifugation (especially if refrigerated before centrifugation), time of day and stage of estrus. In cattle and humans, serum progesterone values have been reported to decline following a meal. Goal of this study was to determine the influence of a meal on serum progesterone in dogs. All procedures were approved by the OSU Animal Care and Use Committee. Client-owned female dogs (n = 7) of various breeds (body weight: 3.4 - 37 kg, mean: 17.65 kg) and ages (2 - 10 years, average 4.9 years) presented to OSU Lois Bates Acheson Veterinary Teaching Hospital for breeding management were enrolled. Dogs were fasted overnight prior to initial blood sampling between 0800 and 0900. Blood was collected into plain red top tubes with no anticoagulant and submitted to laboratory within 20 minutes after sample collection. Following initial sample, dog was fed a commercial dog food (a/d[®] Hills Pet Nutrition, Topeka, KS), 10 - 15 ml/kg body weight (average 13.3 ml/kg). All dogs consumed food readily and no adverse effects were observed or reported by owners. A second blood sample was collected 60 - 75 minutes after feeding (average 68.5 minutes). Serum progesterone was determined by a Siemens Immulite[®] 1000 (Siemens USA, Malvern, PA). Serum progesterone concentrations were lower (p = 0.0005) in post prandial sample in all 7 patients. Average reduction was 28.2% (11.3 - 68.4%), with an average decrease of 0.95 ng/ml (0.14 - 1.41 ng/ml). Two samples of special interest had fasting concentrations of 1.96 and 2.26 ng/ml that dropped postprandial to 0.62 and 0.93 ng/ml, respectively. Body weights of those 2 dogs were 5.8 and 14 kg, respectively. Since LH surge occurs when serum progesterone is in 1 - 3 ng/ml range, interpretation of pre and postprandial samples might be different if the dog was not fasted at the initial sample. However, neither of these 2 dogs tested positive for LH test (Witness[®] LH, Zoetis, Kalamazoo, MI) at first sampling. We recommend that samples for serum progesterone are collected from fasted patients to ensure most consistent results. Cause for decreased serum progesterone concentrations following a meal in dogs has to be determined.

Keywords: Progesterone, feeding, dog, ovulation

Effect of slide type to evaluate motility parameters of frozen-thawed equine sperm using computer aided sperm analysis

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In equine industry, motility assessment is the primary parameter currently used to evaluate sperm quality in an ejaculate. Motility is determined either by visual subjective assessment or objectively via computer aided sperm analysis (CASA) system. Various types of slides and coverslips are used for sperm motility assessment. Two main methods are a fixed coverslip slide (e.g. Leja[®] slide) or a drop coverslip slide (e.g. Cell-Vu[®] slide or glass slide). Both these slides can have a fixed chamber depth of 20 microns. Previous studies in our laboratory demonstrated a negative effect on total and progressive motility with fixed coverslip slides compared to drop coverslip slides for analysis of fresh semen using CASA. Objective was to determine if there is a difference in CASA derived motility parameters of frozen-thawed stallion sperm based on slide/chamber type. We evaluated the postthaw motility of 30 ejaculates of frozen semen. Samples were thawed at 37°C for 30 seconds and placed into a prewarmed 5 ml tube. Concentration was determined using a NucleoCounter and samples were diluted to 30 x 10⁶/ml in 2 ml standard skim milk semen extender. Samples were incubated for 10 minutes and evaluated using the Ceros II CASA system. Then, 5 µl sample was placed in each chamber on either fixed coverslip slide or drop coverslip slide. To avoid any possible influence of an increased incubation time, the first slide type evaluated alternated with every sample. A minimum of 5 fields were selected for each sample and analysis continued until a minimum of 400 motile sperm were examined. Each field was limited to have a maximum number of 150 objects per screen in order to minimize the impact of collisions. Motility parameters evaluated were total motility (TM), progressive motility (PM), path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), linearity (LIN), and straightness (STR). A sperm was considered progressively motile if it had a STR of $\geq 75\%$ and a VAP $\geq 50 \mu\text{m}/\text{sec}$. A Student's paired t-test was used to evaluate the difference in motility values between 2 slides and $p < 0.05$ indicated significance. There was a difference ($p < 0.0001$) for TM and PM between 2 slide types. Both motility values were higher in drop coverslip slides. Average TM for drop coverslip slides was 52% versus fixed coverslip slides was 35%. Average PM for drop coverslip slides was 38% versus fixed coverslip slides was 24%. There was no difference between cell types for VAP, VSL, VCL, STR, LIN, ALH, and BCF. These data agree with previous results in our laboratory obtained with fresh semen. Although drop coverslip slides and fixed coverslip slides utilized 20 micron chamber for motility analysis, these results indicate that slide type has substantial impact on sperm motility and comparison of motility measures between samples using various chamber types is not valid.

Keywords: Equine sperm, motility, CASA, fixed coverslip, drop coverslip

Effect of glycerol concentrations on a new extender for freezing dog semen

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Glycerol is the most commonly used cryoprotectant for frozen semen in several species, because it alters colligative properties of water, decreasing freezing point and increasing sperm survival. Many studies used various concentrations of glycerol for freezing dog semen, with no consensus regarding best glycerol concentration. Aim was to evaluate glycerol concentrations (6, 7, and 8%) in dog freezing extender. Two ejaculates from 12 adult dogs (2 - 5 years) of various breeds were used. Ejaculates were collected by digital manipulation and divided into 3 aliquots, centrifuged at 600 x g for 7 minutes and pellet was resuspended (100×10^6 sperm per ml) in an egg yolk-based extender BotuDog[®] (Botupharma[®]) with 3 glycerol concentrations: 6, 7, and 8% (G6, G7, and G8, respectively). Samples were packaged in 0.5 ml straws (IMV, France) and kept in a controlled temperature refrigerator (Minitub[®]) for 60 minutes at 5°C. Straws were placed in a Styrofoam box and kept 3.5 cm above nitrogen level for 20 minutes, and finally were immersed. Straws were thawed at 76°C for 8 seconds. Sperm kinetics were evaluated using the CASA system (Hamilton Thorne Research - IVOS 12, Beverly, MA). Integrity of plasma and acrosomal membranes, and stability of plasma membrane were determined by flow cytometry (BD LSR Fortessa - Becton Dickinson, Mountain View, CA), at 10 and 30 minutes (T10 and T30), after thawing with incubation at 37°C. Data were analyzed using statistical program Graph Pad 6.0 and Kolmogorv-Sminov test was used for normality. ANOVA followed by Tukey test were used and Friedman test followed by Dunns were used for nonparametric data. Total motility was not different ($p > 0.05$) among groups in T10: G6 (52.5 ± 5.3), G7 (58.7 ± 3.7), and G8 (57.3 ± 5.3) and in T30: G6 (35.1 ± 3.9), G7 (41.4 ± 4.1), and G8 (38.2 ± 3.8), neither progressive motility in T10: G6 (39.5 ± 4.9), G7 (45.4 ± 3.8), and G8 (44.1 ± 4.8) and in T30: G6 (26.3 ± 3.7), G7 (32.0 ± 3.8), and G8 (29.7 ± 3.6). Integrity of plasma and acrosomal membrane was higher in G7 (52.6 ± 3.4) compared to G6 (42.4 ± 3.7) at 10 minutes after thawing and after 30 minutes incubation in G7 (32.3 ± 2.8) compared to G6 (26.4 ± 2.7). We concluded that 7% glycerol in the Botudog[®] freezing extender yielded better results for plasma and acrosomal membrane integrity than 6%, whereas 8% had intermediate results.

Keywords: Cryopreservation, canine, CASA, plasma and acrosomal membrane integrity

Sperm protein reactive with antisperm antibody is immunoexpressed in equine primordial, primary, secondary, and tertiary follicles

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Sperm protein reactive with antisperm antibody (SPRASA) is present in all stages of ovarian follicles in humans, mice, cows, dogs, and cats,^{1,2} and is localized to granulosa cells and ooplasm.^{1,2} Objective was to determine if equine ovarian follicles expressed SPRASA. We hypothesized that SPRASA immune expression would be present in all stages of equine ovarian follicle, and localize to granulosa cells and ooplasm. Routine immunohistochemistry was performed on formalin-fixed paraffin-embedded ovarian sections (4 µm) from 3 adult grade Quarter Horse mares. Briefly, sections were deparaffinized and rehydrated before antigen retrieval was accomplished by heat activation in a sodium citrate buffer. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide and nonspecific binding was blocked using serum-free protein block (Dako #X0909, Carpinteria, CA). Polyclonal antiSPRASA primary antibody was applied to slides at a 1:200 dilution. Universal negative rabbit antibody (Dako #S3022) was applied to adjacent sections to serve as negative control. Horseradish peroxidase-conjugated antirabbit polymer secondary antibody (Immuno Bioscience, #IH-8064-OSU-15, Mukilteo, WA) was applied undiluted to all slides. Peroxidase activity was detected using Nova Red Kit (Vector Laboratories Inc, #SK4800, Burlingame, CA). Slides were counterstained with hematoxylin, dehydrated, and cover slipped. Digital images were captured at 10 and 40 x magnifications (QImaging #QIC-F-M-12-C). SPRASA expressed in pregranulosa cells of primordial, and in granulosa cells of primary, secondary, and tertiary follicles in equine ovaries. SPRASA was not expressed in equine ooplasm. There was no positive staining in negative controls. In US, wild horse and burro populations have drastically exceeded the carrying capacity of public lands where they are managed. With the exception of surgery (removal of ovaries), current methods for sterilizing wild female horses and burros (e.g. porcine zona pellucida or GnRH immunization) are temporary reversible solutions. Immunization against SPRASA may prove to be a permanent nonsurgical sterilant method because it targets all follicles including primordial follicles.

Keywords: Antisperm antibodies, contraceptive, horse, immunohistochemistry, oocyte, SPACA3

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Comparison of two methods to induce acrosome reaction in stallion sperm

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Acrosome reaction (AR) failure in stallion sperm has been associated with pronounced subfertility. Acrosomal function in stallion sperm is typically assessed by sperm reaction to calcium ionophore, A23187 (CaI). This method is considered “nonphysiological,” because sperm viability is greatly reduced. We have recently demonstrated that incubation of stallion sperm under “capacitating-like conditions” (presence of bicarbonate, bovine serum albumin [BSA], and calcium) using a Modified Whitten’s medium containing only lactate as an energy source (Lac-MW) induces spontaneous AR in viable equine sperm (VAR). We compared rate of AR and VAR in stallion sperm induced by the CaI and Lac-MW methods. Two ejaculates each from 4 fertile stallions (n = 8) were collected. Sperm in Lac-MW treatment were washed, resuspended in this medium, and incubated at 38.5°C in 5% CO₂ in air. Sperm in CaI treatment were diluted in INRA-96 extender, treated with 10 µM CaI, and incubated at 38.5°C in air. At 1, 2, 4, and 6 hours of incubation, sperm aliquots were stained using FITC-PSA/propidium iodide (CaI), or FITC-PSA/Fixable Live-Dead Red Stain (Lac-MW), and analyzed by flow cytometry for percentages of viable (%V), total AR (%AR), and viable acrosome reacted (%VAR) sperm. FITC-PSA/Fixable Live-Dead Red stain was used in Lac-MW to reduce artifactual decrease in sperm populations due to agglutination after incubation. Previous data from our laboratory indicated agreement between techniques is high (Bias PSA-PI versus Fixable LD: -4.0%, SD: 5.3). Data were rank-transformed before analysis using a General Linear Model procedure. At all time periods, mean %V was higher in Lac-MW than in CaI (e.g. 1 hour: 62 versus 28%; 6 hour: 59 versus 0%; p < 0.05). Conversely, at all time periods, mean %AR was higher in CaI than Lac-MW (e.g. 1 hour: 40 versus 18%; 6 hour: 95 versus 63%; p < 0.05). Mean %V-AR was different in Lac-MW than CaI starting at 2 h (2 versus 0%), and was notably higher at 4 hour (24 versus 0%) and 6 hour (29 versus 0%; p < 0.05). Use of Lac-MW appeared to be preferable for induction of AR in stallion sperm if viability is desired, since higher VAR were obtained, and treatment did not reduce sperm viability. This suggested that Lac-MW might create a more “physiological” condition for the AR. Differences in %AR and %VAR observed suggested that these 2 methods induce acrosome reaction via different mechanisms. Use of Lac-MW could be useful to investigate the mechanism associated with AR failure in stallion.

Keywords: Stallion sperm, acrosome reaction, viability, lactate, calcium ionophore

Sperm contact time in uterus and endometrial inflammation in mares bred by transrectally guided deep horn artificial insemination

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Sperm elicit an acute uterine inflammatory reaction that resolves within 24 - 48 hours in normal mares. Delay in uterine clearance results in persistent inflammation, alters uterine environment, and decreases embryo survival, resulting in substantial economic losses. To improve embryo survival, uterus is lavaged within the first 4 - 12 hours after insemination. Substantial reduction in pregnancy rate was observed if lavage is performed prior to 4 hours. Deep (uterine) horn transrectally guided artificial insemination (DHAI) is a technique that places sperm close to utero-tubal junction, ipsilateral to the ovulation side. Advantages of DHAI are reduction in volume and/or number of sperm inseminated, and reduction in sperm transport time, with faster oviductal sperm colonization. Since severity of endometrial inflammation is directly related to number of sperm and duration of uterine-sperm interaction, we hypothesized that uterine lavage prior to conventional 4 hours would have an effect on uterine inflammation. Objectives were to determine degree of inflammation at 1 and 4 hours after insemination in mares bred by DHAI and determine pregnancy in mares lavaged earlier than 4 hours. Four warmblood and 3 Thoroughbred mares with normal reproductive histories were used over 14 estrous cycles in a cross-over experimental design. Mares with obvious uterine edema and a dominant preovulatory follicle were induced to ovulate with 1 mg of Deslorelin IM. Within 0 - 6 hours after ovulation, mares were bred by DHAI with 1 x 0.5 ml straw of frozen-thawed semen from 1 of 2 stallions of proven fertility and containing 120 - 160 x 10⁶ total sperm. On the first estrous cycle, mares were randomly assigned for lavage at 1 or 4 hours following DHAI. Mares were rested for a cycle and assigned to opposite group. At lavage, mares were examined for presence and degree of endometrial edema. Presence of uterine fluid and effluent samples were analyzed for presence or absence of PMN's, and PMN numbers. Pregnancy diagnosis was conducted at 12 - 14 days following DHAI. Uterine semen contact time of 4 hours versus 1 hour had a significant effect on degree of uterine edema ($p < 0.046$), presence or absence of PMNs ($p < 0.0009$), and PMN numbers ($p < 0.0006$), but not on amount of accumulated uterine fluid ($p > 0.5$) or pregnancy rate ($p = 0.28$). We concluded that presence of sperm in uterus of normal mare for 1 hour elicited lesser inflammatory reaction compared to 4 hours and pregnancy rate was not affected by early lavage in mares bred by DHAI.

Keywords: Endometritis, deep horn insemination, uterine lavage, pregnancy rate

Inflammatory proteins as novel diagnostic biomarkers for endometritis in mare

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Equine endometritis is a costly, prevalent, and challenging disease for equine practitioners to diagnose, with clinical implications ranging from subfertility to infertility. Currently, gold standard diagnostic test is endometrial biopsy subjected to Kenney-Doig grades; however, time to receive results can be variable. Objective was to test for inflammatory proteins that are previously validated as equine diagnostic biomarkers in uterine low volume lavage (LVL) fluid of mares. We hypothesized that these inflammatory proteins would substantially increase in LVL fluid of mares with documented evidence of endometritis or poor biopsy grade. Light breed mares (n = 29) 3 - 22 years old were used. Each mare had an LVL (250 ml) followed by endometrial biopsy. Endometrial cytology was performed from LVL and biopsy evaluation was graded by a board-certified veterinary pathologist. Mares were either assigned to endometritis group (n = 12), or healthy group (n = 17) based on endometrial cytology (> 1% PMNs), and/or poor endometrial biopsy score (IIB/III). LVL was utilized in a multiplex bead assay (Luminex Corp. Austin, TX) to quantify levels of following biomarkers: IFN γ , IL1 β , IL10, IL17, sCD14, TNF α , chemokine (C-C motif) ligand 2 (CCL2), CCL3, CCL5, and CCL11. Data were analyzed by STATA (College Station, TX). Since data were not normally distributed, a Mann-Whitney U test was performed to compare levels of inflammatory markers between healthy and endometritis mares, with significance set at p value < 0.05. Following inflammatory markers were higher in LVL from mares with endometritis compared to healthy mares: IFN- γ (p = 0.0094), IL17 (p = 0.0296), CCL2 (p = 0.0196), and CCL3 (p = 0.0118). These 4 biomarkers are all proinflammatory cytokines, which orchestrate response of various leukocytes to endometrial changes. Further studies with a larger population are warranted; however, we suggest that proinflammatory markers identified may serve as potential diagnostic markers for endometritis in equine uterine fluid samples. Use of LVL samples for detection of inflammatory biomarkers could provide a less invasive and efficient diagnostic test to practitioners during busy breeding season.

Keywords: Biomarkers, endometritis, equine, low volume lavage

Oxytocin-induced secretion of 13,14-dihydro-15-keto-prostaglandin F_{2α} in mares with prolonged corpus luteum function

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Oxytocin treatment to prolong corpus luteum (CL) function for estrus suppression in mares is replacing use of intrauterine glass ball. Most common oxytocin protocol involves 60 IU of oxytocin treatment given intramuscularly once daily from days 7 - 14 postovulation (day 0). About 70% of treated mares had prolonged CL function lasting for 60 - 90 days. However, a longer duration of CL function would be more favorable. Serial treatment of human chorionic gonadotropin (hCG) during oxytocin-induced prolonged CL function would extend duration of CL function by having a luteotropic effect and/or by inducing ovulation of diestrous follicle(s) resulting in new CL(s) formation that would remain functional for additional 60 - 90 days (i.e. 120 - 180 days of CL function).¹ Although diestrous ovulations were documented during prolonged CL function period, there was no difference between control and hCG-treated mares in duration of CL function. Notably, in some mares, progesterone concentrations decreased abruptly after a diestrous ovulation, suggesting return of endogenous luteolytic mechanism responsible for terminating CL function. Objective was to evaluate the ability of endometrium to secrete prostaglandin F_{2α} (PGF_{2α}) during oxytocin-induced prolonged CL function. Oxytocin treatment was used to prolong CL function in mares that were subsequently assigned to 1 of 3 groups: 50 - 59 days (n = 4), 60 - 69 days (n = 4), or 70 - 79 days (n = 3); groups designated as 50s, 60s, and 70s, respectively). Day 14 mares served as control group (n = 3). Endometrial secretion of PGF_{2α} was evaluated by measuring systemic concentrations of 13,14-dihydro-15-keto-prostaglandin F_{2α} (PGFM) following a single 10 IU intravenous oxytocin bolus (time 0) treatment to mares in all groups. Blood samples were collected at -30, -15, 0, 5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 105, and 120 minutes. Area under PGFM curve was higher in 70s group compared to 50s (p < 0.001) and 60s groups (p < 0.02), with no difference between 70s and control groups. CL function was maintained after oxytocin bolus in all 4 50s mares, 3 of 4 60s mares, and 0 of 3 70s mares. These results provided clear evidence for return of uterine luteolytic mechanism between days 50 - 70 in mares with prolonged CL function.

Keywords: Equine, mare, estrus suppression, oxytocin, corpus luteum, PGFM

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Pharmacodynamics of clomiphene citrate in cyclic mares

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Clomiphene citrate (CC), a selective estrogen receptor modulator (SERM), is an orally administered reproductive drug that improved follicular recruitment and development primarily in human but also in bovine, ovine, and lagomorph species. Experimental data from mare after CC administration are slim; however, a 40 year-old, anecdotal case report describes some success after use of CC in 3 anestrus mares. We hypothesized that administration of CC in mare would result in an increased number of ovarian follicles. Objectives were to quantify AMH, LH, and FSH serum concentrations, to characterize endometrial edema, and changes in follicular dynamics between treated and control mares. Light-breed mares (n = 12) of 6 - 18 years were utilized from mid-July to early August. Mares (n = 6) had oral treatment of CC (500 mg) once daily for 5 consecutive days and control mares (n = 6) had oral placebo. Blood samples for AMH concentrations were collected on days 1, 3, 6, and 9. Blood samples for FSH and LH concentrations were collected prior to first dose of drug and then once daily through treatment period. Transrectal ultrasonography and follicle mapping were performed in both treated and control mares. Statistical analyses were done using two-way repeated measures ANOVA, with $p \leq 0.05$. Administration of CC did not induce changes in follicle numbers ($p > 0.05$). Plasma AMH concentrations did not differ between experimental groups; however, mean concentrations relative to time were different ($p = 0.009$). There were no differences for plasma FSH or LH concentrations, however, daily mean plasma LH varied ($p < 0.001$) with time within experimental groups. Endometrial edema scores were higher ($p = 0.041$) in mares treated with CC than in control mares, after allowing for time effect. In conclusion, daily administration of CC for 5 consecutive days did not affect follicle numbers or elicit substantial endocrine response. In humans, efficacy of CC treatment in correcting ovulatory dysfunction derives from its blocking effect on hypothalamic estrogen receptors. That, in turn, causes a significant release of FSH and LH. In contrast, in the present study, CC treatment had substantial estrogenic effects in uterus by inducing endometrial edema. Thus, it remains to be elucidated whether CC in mare has ability to modulate FSH and LH secretion. Pharmacokinetics analyses of blood samples are pending, and could provide a basis for revised protocols for CC administration.

Keywords: Equine, LH, FSH, AMH, estrogen, follicle dynamics

Effect of mycobacterium cell wall fraction on histological, immunological, and clinical parameters of equine uterine involution

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Maintaining yearly foal production is important for economic success of broodmares and requires breeding to occur as quickly postpartum as possible. First estrus occurs within 5 - 20 days postpartum, during uterine involution uterus (repair from tissue alterations of pregnancy and parturition). Attempts to hasten uterine involution have had minimal success. Mycobacterium cell wall fraction (MCWF), an immunomodulator, reduced bacterial growth and altered aspects of immune response to breeding; however, it is unknown if MCWF hastened uterine involution. Objectives were to: 1) investigate effect of MCWF on tissue remodeling; and 2) assess effect of MCWF on cell-mediated immunity of uterus. We hypothesized that MCWF treatment in postpartum would hasten uterine involution. Pregnant mixed breed mares (n = 10) were evaluated postpartum. Control mares (n = 4) received 1.5 ml LRS intravenously on day 1 postpartum and again 7 days later and experimental mares (n = 6) received 1.5 ml Settle[®] (MCWF) intravenously on day 1 and again 7 days later. All mares were assessed every 3 days for clinical, immunological, and histological parameters until day 15 postpartum. Clinical parameters were assessed via transrectal ultrasonography and included assessment of ovarian activity, uterine fluid retention, and uterine wall diameter, in addition to obtaining endometrial culture. Immunological parameters included endometrial biopsies for qPCR for expression of various cytokines (IL1 β , IL1RN, IL4, IL6, IL8, IL10, TNF, IFN γ , and GM-CSF) and endometrial cytology. Histological parameters were assessed on formalin-fixed endometrial biopsies and variables included retention of microcotyledons, endometrial glands dilation, and inflammation of mucosa, stratum compactum, and spongiosum. Data were analyzed using SAS 9.4, utilizing a Mixed model for repeated measures, with treatment as a random effect. All posthoc analyses used Tukey's HSD test. Involution was considered complete by day 15 postpartum in all mares, and day postpartum had significant effect on all parameters investigated, indicating involvement of immunological process in uterine involution. Treatment with MCWF decreased severity of bacterial growth, in addition to time to obtain clean culture. MCWF treatment increased expression of proinflammatory cytokines, namely, IL1 β , IFN γ , and TNF. Whereas treatment effect was minimal, there was histological evidence for decreased mucosal inflammation in MCWF-treated mares. In conclusion, uterine involution is heavily regulated by immune system. Additionally, MCWF had bactericidal effect on postpartum mare; this may be due to increases in proinflammatory cytokine expression. Further studies are needed to determine whether this immunomodulator will improve first estrous cycle fertility in postpartum mares.

Keywords: Mare, uterine involution, mycobacterium cell wall fraction, postpartum, immunomodulator

Transcriptomic analysis of equine chorioallantois reveals key regulators and pathways involved in ascending placentitis

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Although placentitis is the costliest disease in equine industry, its current standard treatment is not satisfactory and progress is slow in identifying efficient placentitis prevention, diagnostics, and treatment protocols. Therefore, a faster, more holistic view of molecular mechanisms underlying equine placentitis holds potential for development of new diagnostic tools and therapies to forestall placentitis-induced preterm labor. Our hypothesis was that characterizing transcriptome of equine chorioallantois (CA) during placentitis in comparison to pregnancy matched controls would elucidate key regulators and molecular mechanisms triggering equine placentitis. Chorioallantois samples were collected after euthanasia at ~ 290 days of pregnancy in mares with experimentally induced placentitis (placentitis group, n = 6) and uninoculated mares (control group, n = 6). Next generation RNA sequencing was performed with Illumina NovaSeq6000 and reads mapped to EquCab3.0 (STAR-2.5.2b). Differentially expressed genes (DEGs) were identified with Cuffdiff-2.2.1 (FDR < 0.05). Our study identified 2953 DEGs (2028 upregulated and 925 downregulated) in CA during placentitis compared to controls. Pathway analysis of DEGs revealed that these genes were involved in relevant pathways, such as inflammatory signaling (inflammation mediated by chemokine and cytokine signaling pathway, interleukin signaling, toll-like receptor signaling, T cell activation, and B cell activation), angiogenesis-related pathways (vascular endothelial growth factor signaling and angiogenesis pathway), cytostructural integrity (integrin signaling pathway), and apoptosis signaling pathway. Upstream regulator analysis revealed central role of toll-like receptors (TLR2, TLR3, TLR5, and TLR7) in triggering inflammatory signaling after placental infection, and this consecutively resulted in placental inflammation and immune cells chemotaxis. Increased leukocytic infiltration in CA was associated with upregulation of matrix metalloproteinase (MMP1, MMP2, and MMP9) with subsequent extracellular matrix (ECM) degradation and apoptosis, as reflected by upregulation of several apoptosis-related genes such as caspases (CASP3, CASP4, CASP7, and CASP10) that are believed to be implicated in placental separation during disease course. Additionally, activation of TLRs was associated with upregulation of a wide array of transcription factors and TLRs dependent downstream molecules in the inflammatory cascade. This in turn was associated with downregulation of transcripts coding for proteins essential for placental steroidogenesis (e.g. SRD5A1, AKR1C1), angiogenesis (e.g. VEGFA), nutrient transportation (e.g. GLUT12), and upregulation of hypoxia-related genes (e.g. HIF1A) that could explain placental insufficiency during placentitis. First time key regulators and mechanisms underlying placental inflammation, separation, and insufficiency during equine placentitis were characterized. Findings might lead to development of efficacious therapies by targeting key molecules and pathways.

Keywords: Pregnancy, placentitis, chorioallantois, mare, RNA-sequencing

Comparison of glucose and lactate concentrations between healthy equine and mule foals, and of an automated laboratory analyzer and Accutrend® Plus system

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Mules are hybrids resulting from breeding between female horses (*Equus caballus*) and male donkeys (*Equus asinus*). Information about physiological adaptation of newborn mules is scarce in literature. Profiles of blood glucose (GLUC) and lactate (LACT) concentrations are important to evaluate neonates, as they indicate metabolic dysregulations, disease severity, and prognosis. Thus, fast and affordable GLUC and LACT evaluations at farms using portable analyzers would be valuable. Additionally, it is important to know the pattern of variation of these parameters to differentiate healthy and compromised equid neonates. We hypothesized that portable devices measure glucose and lactate concentrations accurately, that these parameters differ between equine and mule foals, and vary over time. Aims of this study were to: 1) compare GLUC and LACT blood concentrations between equine and mule foals during their first 720 hours of life; 2) evaluate LACT and GLUC profiles from birth to 720 hours of life; and c) establish the correlation of LACT and GLUC concentrations using Accutrend® Plus system (ACP) with whole blood and Randox Daytona automated analyzer (AUTO) with plasma in both species. Healthy equine (n = 16) and mule foals (n = 15) were used and blood samples were collected immediately (T0), 1 (T1), 6 (T6), 12 (T12), 24 (T24), 168 (T168), and 720 (T720) hours after birth. GLUC and LACT concentrations were evaluated with an AUTO and with an ACP. Data were analyzed by repeated measures using PROC MIXED and intraclass coefficient correlation (ICC) between 2 analyzers was calculated. Overall, GLUC concentrations evaluated with AUTO were different between species; LACT concentrations were higher ($p < 0.05$) in equine foals when compared to mule foals (2.14; 1.01 - 5.99 and 1.83; 0.61 - 9.04 mmol/l, respectively). There was no difference in GLUC and LACT results evaluated with ACP for equine and mule foals. Pattern of variation of GLUC evaluated with both analyzers for equine and mule foals changed during first 720 hours of life, with an increase in GLUC concentrations soon after nursing and then a decrease to baseline levels. LACT pattern of variation was similar for all foals, with both analyzers detecting higher LACT values at T0 (4.05; 1.91 - 9.04 mmol/l), with a progressive decrease until T720 (1.21; 0.61 - 5.88 mmol/l). The ICC for equine and mule foals between AUTO and ACP for GLUC concentrations was low and was moderate for LACT concentrations ($ICC < 0.90$). We concluded that GLUC and LACT concentrations changed during first 720 hours after birth in mule and equine foals, with GLUC values differing between species. Additionally, we recommend standardization of the ACP with specific samples before clinical use for GLUC and LACT analysis.

Keywords: Neonate, hybrid, equine, hematological parameters, critical ill, prognosis

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Effect of ejaculation frequency, prostaglandin F_{2α} and cold storage on canine semen yield and postthaw quality

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Number of sperm obtained from a single semen collection can vary widely among dogs, often requiring multiple ejaculates to produce a single breeding dose of cryopreserved sperm. Alternative collection techniques and schedules have been reported to increase sperm yield, however, a direct comparison of these methods is warranted. Objective of this study was to determine most effective collection regimen to increase total number of sperm available for cryopreservation without compromising postthaw motility parameters. We hypothesized that multiple collections and administration of prostaglandin F_{2α} (PGF_{2α}) could be used to increase sperm yield without detrimental effects to postthaw semen quality. Sexually mature male dogs (n = 5) were all subjected to following 5 collection protocols in a random order: 1) 1 collection (control); 2) 2 collections 1 hour apart; 3) 2 collections 24 hours apart; 4) 2 collections 48 hours apart; and 5) 1 collection 20 minutes after PGF_{2α} (Dinoprost, 0.05 mg/kg, IM). Total sperm count, total motility (TM) and progressive motility (PM) were assessed for each ejaculate using computer assisted sperm analysis (CASA). Semen was either frozen immediately after collection (Protocols 1 and 5) or stored at 4°C, pooled with a second ejaculate (Protocols 2, 3, and 4), and then frozen using a Tris-egg yolk media. Dogs were allowed 7 days of sexual rest between protocols. Frozen samples were evaluated using CASA and flow cytometry to determine TM, PM, viability (VIA) and acrosome integrity (ACR). Data were analyzed using a general linear mixed model and Tukey's posthoc for pairwise mean comparisons. Significance was set at p < 0.05. All collection protocols resulted in a higher sperm yield compared to control. Postthaw semen parameters (presented in percent) were similar between control and Protocols 2 and 5 (TM: 50.4 ± 5.1, 49.4 ± 5.1, 53.8 ± 5.1, PM: 36.8 ± 4.7, 35.4 ± 4.7, 40.2 ± 4.7, VIA: 61.9 ± 3.4, 65.3 ± 3.4, 61.7 ± 3.4, ACR: 23.2 ± 2.7, 18.1 ± 2.7, 20.4 ± 2.7), respectively. Protocol 4 resulted in poor postthaw semen parameters (TM: 30 ± 5.1, PM: 20.4 ± 4.7, VIA: 49.8 ± 3.4, ACR: 29.8 ± 2.7). Protocol 3 had intermediate results for all semen parameters (TM: 37.2 ± 5.1, PM: 24.4 ± 4.7, VIA: 55.2 ± 3.4, ACR: 26.6 ± 2.7). In conclusion, 2 collections performed 1 hour apart with 1 dose of PGF_{2α} dramatically increased sperm yield and also maintained postthaw quality. Cooled storage before cryopreservation was increasingly detrimental with increasing time between collections. We provided 2 alternative semen collection protocols to practitioners to maximize number of breeding doses cryopreserved in 1 visit. Further, this would enhance client satisfaction while also minimizing costs and veterinary visits.

Keywords: Canine, spermatozoa, yield, cryopreservation, prostaglandin

Seroprevalence of canine brucellosis in the southeastern United States

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There are four *Brucella* strains of zoonotic importance: smooth strains, i.e. *Brucella abortus* (*B. abortus*), *B. melitensis*, *B. suis*, and a rough strain, i.e. *B. canis*. Dogs can serve as hosts for all four zoonotic strains; however, standard serologic testing in dogs is limited to the identification of only *B. canis* antibodies. Well known to be transmitted venereally, *Brucella* are also readily shed in urine, feces, saliva, milk, and respiratory secretions, and can be easily transmitted through close contact. There is no approved treatment for the elimination of *Brucella* from infected animals. Human infection is a nationally notifiable condition, as treatment is not always effective in clearing the organism, resulting in recurrent infection. Therefore, preventing spread of disease is paramount for public health. The goal of this pilot study was to estimate the number of dogs with circulating antibodies to any of the four zoonotic strains of *Brucella* by sampling multiple subpopulations of hog hunting dogs, dogs presenting for routine spay and neuter, and imported animals throughout the southeastern United States. We hypothesized that the seroprevalence of smooth strains of brucellosis would be higher in imported and hog-hunting dog populations than dogs presenting for routine spay and neuter, and that the seroprevalence of the rough strain of brucellosis (*B. canis*) would be similar across all groups. To date, serum has been harvested from 65 client owned dogs (30 hog hunting and 35 companion dogs) and 19 shelter owned dogs of various ages, parities, and breeds. Reproductive tissues, semen and/or vaginal swabs were collected when available. Brucellosis serology was performed using the Canine *Brucella* Slide Agglutination test (SAT), *Brucella canis* Agar Gel Immunodiffusion II test (AGID), *Brucella abortus* Card Agglutination test (CAG), and *Brucella abortus* Fluorescence Polarization Assay (FPA). To date, among 84 samples collected and assayed, one shelter dog tested positive for a rough *Brucella* strain via AGID. An imported dog tested positive for both a rough and a smooth strain of brucellosis via SAT, AGID and FPA, and two client owned dogs tested positive for a smooth *Brucella* strain by a National Veterinary Services accredited laboratory via *B. abortus* plate and card agglutination. Three attempts at culturing the organism from reproductive tissue samples of seropositive animals yielded contaminant growth only. All other samples (n = 80) were seronegative. Although this project is still ongoing, at this time, 4 of 84 samples (5%) tested positive for at least one strain of *Brucella*, with 3 of 4 positive samples (75%) testing positive for a smooth *Brucella* strain. All hog-hunting dogs sampled were seronegative. Our preliminary results support an overall low seroprevalence rate for brucellosis, with most samples testing positive for a smooth *Brucella* strain, thus, supporting the need for a commercially available, validated test for the smooth strains in canines. To the authors' knowledge, this is the first study evaluating the seroprevalence of the smooth strains of brucellosis in dogs in the US.

Keywords: Canine brucellosis, rough strains, smooth strains, seroprevalence

Effect of polyacrylamide hydrogel injection into reproductive tract of mares

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Urogenital problems are common in older, multiparous broodmares. Vesicovaginal reflux (VVR) or “urine pooling” is a condition where urine refluxes from urethra and accumulates within cranial vagina. Currently, treatment for this condition involves medical management and surgery. Caudal relocation of transverse fold or extension of urethra are surgical procedures utilized to treat VVR. Currently, injectable therapies designed to bulk tissue surrounding transverse fold have not been utilized to treat VVR. Use of a polyacrylamide hydrogel to alter vestibulovaginal fold anatomy may be an adjunctive treatment option for VVR in mares. Polyacrylamide hydrogel is a nontoxic and nonimmunogenic biocompatible, nonabsorbable polymer gel that consists of 2.5% cross linked polyacrylamide and 97.5% sterile water.¹ This compound has been utilized safely for soft tissue augmentation and urinary incontinence in women for several years.² Objective was to evaluate Synamid[®] (Contura International A/S, Sydmarken 23, 2860 Soeborg, Denmark) injection safety into vestibulovaginal mucosa of normal mares. Ten mares (aged 7 - 15 years) were utilized. Prior to procedure, physical examinations and thorough reproductive evaluations were performed. Subjects were administered 3 ml of saline and 3 ml of Synamid[®] in separate sites, randomly assigned to 1 cm to either right or left of midline (urethra) in transverse fold. Mares underwent daily physical examination, transrectal palpation and ultrasonography, vaginal speculum examination, and digital examination of vagina for first 7 days, then at days 15 and 30. Daily physical examinations revealed no systemic abnormalities in all mares treated. Hematoma formation at injection site occurred in 7/10 mares, which was observed starting at 24 hours postinjection. Hematomas persisted for an average of 3 days. No pain was elicited on palpation of vestibulovaginal fold in any mares. A palpable bulge was present on Synamid[®] injection site for 30 days in 9/10 mares. In conclusion, no severe complications were observed with injection of Synamid[®] into transverse fold in mares. There is a potential for this long lasting product utilization in “urine pooling” mares to augment vestibulovaginal anatomy and in mares with issues of vulvar incompetence. Further research is needed to determine this product’s efficacy in treating these conditions.

Keywords: Mare, urine pooling, polyacrylamide hydrogel

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Uterine expression of leptin, RhoA and Rho associated kinases in dogs with primary uterine inertia and with obstructive dystocia

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Primary uterine inertia (PUI) is the most frequent dystocia in the bitch, but its etiology is still unclear. Knowledge about underlying functional and molecular changes in the uterus is very scarce. Obesity and high concentrations of leptin (major adipose derived hormone) are associated with complications of labor in women, and obese bitches are at increased risk of dystocia. In pregnant dogs, leptin is also produced by the uterus and placenta, so it may exert a negative paracrine/autocrine effect on uterine contractility during parturition. We hypothesized that high uterine leptin concentrations would adversely affect RhoA/Rho kinase pathway, which is involved in calcium sensitization, leading to decreased uterine contractions in dogs with PUI. Bitches presenting with dystocia (n = 18) were assigned to PUI (n = 11) or obstructive dystocia (OD) group (n = 7). Dogs in OD group were still showing strong contractions and were designated as controls. Dogs that received ecbolic or tocolytic medication were excluded. During cesarean section, a full thickness uterine biopsy was collected at an inter-placental site. Relative gene expression (RGE) of Lep, Lep receptor (LepR), RhoA and its effector kinases (ROCK1, ROCK2) was analyzed by real-time TaqMan qPCR. Immunohistochemistry or *in situ* hybridization was used for protein or mRNA localization, respectively. One-way ANOVA was used to compare uterine RGE between groups with body weight as covariate. Statistical significance was $p < 0.05$. Dogs were not obese (body condition score recorded for 14 dogs 3-6/9). Lep expression did not differ between groups. LepR mRNA levels were below detection limit in 5 PUI dogs and in all OD dogs. RhoA expression did not differ, but uterine ROCK1 and ROCK2 mRNA levels were higher in PUI dogs ($p = 0.010$ and $p = 0.039$, respectively). Lep, RhoA, ROCK1, ROCK2 protein, and LepR mRNA were all localized in myometrium, and staining intensity appeared similar between groups. Protein and mRNA signals were also present in endometrium and blood vessels. In conclusion, despite similar uterine leptin gene expression, undetectable LepR mRNA levels in OD dogs might indicate decreased responsiveness to potentially negative effects of leptin. However, ROCK1 and ROCK2 gene expression was lower in OD than in PUI dogs, which may be a physiological result of long-lasting, strong uterine contractions, and as such, could be an indication of abnormal progression of labor and insufficient uterine contractions in dogs with PUI.

Keywords: Uterine inertia, parturition, uterus, contractility, canine

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Efficacy of a silicone Y design intrauterine device as horse contraceptive in captive breeding

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Due to continued population growth on western rangelands in US, there are now ~ 70,000 feral horses administered to by Bureau for Land Management (BLM) in an area where range ecologists have established 27,000 as desired population size. Therefore, need for an effective contraceptive for feral horses on western range remains a BLM priority. Goal was to find a means of reducing unwanted pregnancies within US feral horse population, while maintaining reversibility so that genetic diversity can be maintained within population. Multiple O Ring designs were deemed unsuccessful due to their inability to resist uterine expulsatory forces in face of immediate and subsequent breeding behavior. We hypothesized that coadministration of long acting progesterone at IUD insertion would increase IUD retention by preventing these immediate aftereffects. Furthermore, discovering that a fundus seeking design in human IUDs ameliorates normal uterine expulsion forces, we formulated an IUD 'Y' design to test for a desired minimum $\geq 75\%$ retention rate. This current study compared 50 and 60 durometer (an indicator of material hardness) 'Y' design IUDs, using 2 breeding pods of 10 mares and 1 stallion in each group. In each pod, 5 mares received a 50 durometer IUD and 5 mares received a 60 durometer IUD. Every other mare received an IM injection of saline or long acting P₄. After early data indication that 60 durometer 'Y' shaped IUDs had a higher retention rate, 50 durometer IUDs were replaced with 60 durometer IUDs. Over next 2 years, all mares in both breeding pods were then monitored by transrectal ultrasonographic examination every 2 weeks to determine IUD retention and pregnancy rates for those mares that lost their IUDs. Endometrial biopsies were obtained, and a Kenney Biopsy grade assigned, before and after research period in efforts to assess IUD impact on endometrial health. Five of 20 mares expelled their IUD (75% retention rate) with each of these 5 mares becoming pregnant. Seven of 20 had no change in Kenney biopsy grade, 4 mares worsened by 1 grade, 5 mares worsened by more than one grade, and 4 mares improved by 1 grade. After 4 cycles following removal of IUDs, 12 mares became pregnant. Based on histopathologic and pregnancy results, mares that failed to become pregnant not due to a worsening endometrial grade but possibly due to involvement of other intrinsic factors. Y design IUD met intended parameters and achieved study goals.

Keywords: Intrauterine device, feral horse, Y design, endometrial grade, fertility

Canine brucellosis: serologically diagnosed positive cases

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Canine brucellosis is a devastating disease as it carries zoonotic potential and there are no effective treatments to clear infections in affected animals. Thus, animals that test positive are commonly euthanized. It is thought to primarily affect intact animals and can be readily transmitted through oral or venereal route. In past few years, number of inquiries for advice on managing positive animals has increased sharply. Goal was to evaluate serologically positive *Brucella canis* submissions. We hypothesized that number and proportion of positive cases would vary by year, source of submission, and patient demographics. Results from samples submitted for *B. canis* slide/Agar Gel Immunodiffusion (AGID II) tests to Cornell Animal Health Diagnostic Center from 2014 - 2019 from US were included in this retrospective study. Submissions were classified as either from a referral laboratory or from a veterinarian. Patient demographics (age, breed, and gender), source (referral laboratory or veterinarian, and state/region), and year were evaluated for association with positive serological diagnosis using logistic regression analysis SAS (v9.4) proc logistic. All variables tested were associated with positive serology ($p < 0.0001$) and were retained in final model. Number and proportion of positive cases were: 231 (16.8%) in 2014, 199 (10.3%) in 2015, 232 (11.4%) in 2016, 217 (11.8%) in 2017, 414 (14.8%) in 2018, and 293 (10.0%) in 2019. Referral laboratory submissions ($n = 882$; 17.4%) had more positive submissions compared to veterinarian submissions ($n = 704$; 9.0%). Animals between 1 - 6 years of age and mix breeds were more likely to be serologically positive compared to pure breeds. Most (83.0%) submissions were from intact animals, but proportion of positive animals was higher in spayed (33%) versus intact females (9%) and castrated (28%) versus intact males (8%). Number and proportion of serologically positive submissions were significantly different among South (14%), West (12%), Midwest (9%), and Northeast (3%) regions. Many submissions were likely prescreened by card agglutination test; thus, results should be interpreted with this in mind. Years 2018 and 2019 had higher number of positive submissions compared to 2014 - 2017. Submissions from young, mixed-breed animals were more likely to test positive, and intact animals had higher numbers but lower proportions of positive cases. Regional difference was also observed. We recommend testing breeding animals and semen to reduce spread of disease.

Keywords: Canine brucellosis, AGID II test

Use of intravaginal progesterone releasing device and estradiol 17 β is equivalent to estradiol 17 β and long acting progesterone in synchronizing acyclic embryo surrogate mares

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Long-acting progesterone (LA P₄) is the most common hormone used to synchronize acyclic surrogate mares for embryo transfer (ET). However, mares synchronized with LA P₄ not used for ET for various reasons (e.g. donor yielding a negative flush or nonviable embryo) do not respond to a resynchronization in a short time, likely due to residual LA P₄ in plasma. This results in economic losses to ET programs due to days that the surrogate mare is not being utilized. Intravaginal progesterone releasing devices (IPRD) for cattle have been used in transitional mares to hasten cyclicity. However, IPRD has not been tested to timely synchronize ET surrogate mares. This study aimed to assess serum progesterone (P₄) concentrations, pregnancy rates after ET and pregnancy losses of embryo surrogate mares synchronized with IPRD. In Experiment 1, crossbred acyclic mares (n = 12) received estradiol 17 β ([17 β beta, Botupharma, Brazil] 10, 20 and 10 mg, respectively, on 3 consecutive days. At 24 hours after the last estradiol injection, an IPRD (Sincrogest, Ouro Fino, Brazil) containing 1 g of natural P₄ was inserted vaginally and kept for 9 days. Blood samples were collected daily for 11 consecutive days and P₄ concentrations assessed using RIA. Experiment 2 was conducted for 2 breeding seasons (2016 - 2018), with crossbred embryo surrogate mares, randomly assigned to 3 groups: 1) cyclic mares (n = 43) having ovulation confirmed after induction (follicle \geq 35 mm) with histrelin acetate (250 μ g, IM, Strelin, Botupharma); 2) acyclic mares (n = 57) treated with estradiol 17 β for 3 days (as above) and then given a single dose of LA P₄ (P4-300, 1.5 g Botupharma); or 3) acyclic mares (n = 57) treated with estradiol 17 β for 3 days and then given an IPRD (Sincrogest). Day-8 embryos were transferred 4 - 8 days after ovulation or P₄ treatments, using a nonsurgical, transcervical technique. Mares in Group 3 had IPRD removed immediately before ET, and immediately after ET, a new IPRD was inserted. Pregnancy diagnosis was performed at 5 days after ET, and 30 and 60 days later. Once pregnancy was first confirmed, mares in Groups 2 and 3 received weekly injections of LA P₄ (1.5 g) until 120 days of pregnancy. Mares in Group 3 had the device removed 3 days after pregnancy diagnosis. Data on P₄ concentrations were analyzed using ANOVA-RM and Tukey posthoc and data on pregnancy rates and losses were compared by multivariate logistic regression (p < 0.05). In Experiment 1, P₄ concentrations significantly increased from IPRD insertion (0.5 \pm 0.2 ng/ml) to next day (15.8 \pm 3.1 ng/ml), and then maintained satisfactory P₄ concentrations deemed suitable for successful establishment of pregnancy until day 9 (7.7 \pm 2.3 ng/ml). After IPRD removal, P₄ concentrations were reduced significantly to baseline (0.78 \pm 0.4 and 0.67 \pm 0.3 ng/ml on days 10 and 11, respectively). For Groups 1-3, there was no difference in pregnancy rates (64, 66, and 69%, respectively) or pregnancy losses by 60 days of pregnancy (17, 13, and 10%). In conclusion, IPRD used herein resulted in a rapid increase in P₄ and sharp decline, upon its insertion and removal, respectively. In addition, this device can be used as a compatible alternative to LA P₄ to synchronize acyclic embryo surrogate mares.

Keywords: Equine, recipient mare, embryo transfer, fertility, hormonal therapy

Effect of age on follicle stimulating hormone receptor expression in ovine endometrium

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Follicle stimulating hormone receptors (FSHR) are present predominately in granulosa cells of secondary and preovulatory follicles. In addition, FSHR are present within ovine endometrium.¹ In humans, ovarian FSHR expression decreased with age.² Objective of this study was to compare endometrial FSHR expression among ewes < 9 months of age (prepubertal/peripubertal; n = 6), 1.5 - 2 years of age (mature; n = 4), and > 3 years of age (aged; n = 4). We hypothesized that similar to human ovary, ovine endometrial FSHR expression would decrease over time. Intracaruncular endometrial samples were collected from crossbred ewes (n = 14) at slaughter. Age of ewe was determined by dental eruption and wear. Ovine ovary served as positive control. Samples were fixed in 10% formalin, embedded in paraffin, and sectioned (5 µm) onto charged slides. Sections were deparaffinized in xylene and rehydrated in graded ethanol series. Heat-induced antigen retrieval was performed with sodium citrate. Endogenous peroxidases were blocked with 3% hydrogen peroxide and nonspecific binding was blocked with a serum-free protein block. Rabbit antihuman FSHR (F-3929, Sigma Aldrich) was applied to tissues at 1:200 dilution and universal rabbit negative agent was applied to an adjacent tissue section to serve as control. Horseradish peroxidase-conjugated polymer antirabbit IgG was used as secondary antibody, followed by treatment with NovaREDTM peroxidase substrate, and counterstained with hematoxylin. Slides were dehydrated in graded ethanol series, passed through 3 xylene solutions, and then coverslipped. Slides were evaluated by a blinded observer (HH) at 400 x magnification with a Leica DM4000B microscope. Intensity of FSHR expression was quantified from no staining (score of 0) to very strong staining (score of 4) for various endometrium compartments from 3 randomly selected fields per ewe.¹ Data on FSHR expression between age groups were analyzed with one-way ANOVA. Significance was defined as $p < 0.05$. Although FSHR was expressed in endometrial luminal epithelium, superficial and deep glandular epithelium, superficial and deep blood vessels, and superficial and deep stroma in both age groups, there were no differences ($p > 0.05$) in expression among age groups. It is not clear what role FSHR plays in ovine endometrium, but it may regulate mucin-1 expression that is involved in early pregnancy establishment.³

Keywords: Immunohistochemistry, intracaruncular, sheep, uterus

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Acupuncture reduces milk N-acetyl beta-D-glucosaminidase in dairy cows with mastitis

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Acupuncture (AP) is used by dairy veterinarians as a complementary treatment for various conditions.¹ NAGase is a lysosomal glycosidase released from mammary epithelial cells during mastitis.² Objective was to determine if AP reduces mammary inflammation. We hypothesized that dairy cattle with subclinical mastitis treated with an intramammary antibiotic and AP would have lower somatic cell count (SCC) and milk N-acetyl beta-D-glucosaminidase (NAGase) concentrations compared to cattle treated with only an intramammary antibiotic. Lactating dairy cows at Oregon State University Dairy Research Center were selected (cows with SCC score of SCC > 500,000 cells/ml) based on Dairy Herd Improvement Association reports. A California Mastitis Test (CMT) determined the quarter that had high SCC (CMT score > 1). Pretreatment milk samples (pre) were collected for SCC scoring and NAGase concentrations. SCC was measured by DeLaval cell counter (Tumba, Sweden) and NAGase concentrations were determined by an ELISA kit (#MBS090625, MyBioSource, San Diego, CA). Cows were treated with an intramammary infusion of ceftiofur hydrochloride (Spectromast LC[®], Zoetis, Parsippany, NJ) and randomly assigned to AP group (n = 7) and nonAP (control) group (n = 7). Both group cows were restrained for 30 minutes in a head catch 4 times, 12 hours apart. Six AP points were used (bladder [BL] 30, BL 30-1, BL 49, kidney [KI] 10, conception vessel [CV] 2, CV 3). Posttreatment milk samples (post) were collected 14 days after presample collection. Pre CMT scores, NAGase concentrations, and SCC were compared to post scores within and between groups. Significance was defined as p < 0.05. Both control and AP groups had reduced post CMT scores (pre: 1.41 ± 0.49; post: 0.42 ± 0.66; p = 0.01 and pre: 1.21 ± 0.39; post: 0.57 ± 0.79; p = 0.039, respectively) but there was no significant change in SCC in either group. In AP group but not in control group, NAGase concentrations were reduced (pre: 19.70 ± 3.65 U/L; post: 16.70 ± 2.05 U/L; p = 0.04 and pre: 25.60 ± 12.90 U/L; post: 20.40 ± 6.04 U/L; p = 0.20, respectively). Reduction in NAGase provided evidence for AP as a complementary treatment.

Keywords: Complementary treatment, California mastitis test, somatic cell count, subclinical

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Dominant follicle removal prior to superovulation

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Superovulation enables cattle producers to reach reproductive, financial, and genetic goals. Although knowledge of follicular development has improved, number of transferable embryos per collection has not been determined, leading to a high degree of unpredictability. Follicle stimulating hormone (FSH) is a major cost of embryo transfer, and treatment must occur coincident with an endogenous FSH surge for effective superovulation and embryo recovery, which has not improved substantially in many years. Following dominant follicle ablation, an FSH surge and associated follicular wave can be predicted and managed, which may lead to more consistent embryo collections and more transferable embryos. Aim of this field trial was to evaluate dominant follicle ablation prior to superovulation with a minimal dose of FSH. Cycling beef cattle, at random stages of estrous cycle, were subjected to transvaginal ultrasound-guided aspiration of all follicles (> 5 mm). Following aspiration, PGF_{2a} (25 mg) was administered and a CIDR was placed. Approximately 48 hours later, Folltropin-V administration began and was given twice daily (am and pm). On third day of FSH administration, PGF_{2a} was administered again and CIDRs were removed that evening. Cattle were inseminated at estrus. One week later, embryos were collected and corpora lutea (CL) were counted using transrectal ultrasonography. Data were analyzed using ANOVA. Neither number of follicles ablated, nor diameter of ablated follicles had any statistically detectable effect on embryo recovery; however, cattle ($n = 24$) with a CL > 20 mm at ablation, tended ($p = 0.107$) to produce more transferable quality embryos (mean \pm SEM; 7.8 ± 1.1) than those with a CL ≤ 20 mm (5.6 ± 0.6). Cattle ($n = 35$) given ≥ 10 ml of FSH had similar number of total ova (11.3 ± 1.3), transferable embryos (6.2 ± 0.9), and CL (14.2 ± 0.9) compared to cattle ($n = 28$) given < 10 ml of FSH (12.3 ± 1.1 , 6.3 ± 0.6 , 15.1 ± 1.0 , respectively). Top quartile of embryo donors ($n = 15$) had a CL diameter = 22.2 ± 5.0 mm, ≈ 8 follicles ablated per ovary, and largest ablated follicle was 11.5 ± 0.6 mm. Donors from this group yielded following numbers: total ova (13.1 ± 1.3), transferable embryos (11.2 ± 1.1), degenerate embryos (1.5 ± 0.4), and unfertilized (0.4 ± 0.2). Total dose of FSH for these donors was 9.9 ± 0.7 ml (i.e. 0.9 ml per transferable embryo). In conclusion, dominant follicle removal prior to superovulation required less exogenous FSH to achieve acceptable embryo recovery. This approach also facilitated acceptable results from consecutive embryo recoveries. These results indicated that ablation of follicles (> 5 mm) in cycling mid-diestrus beef cattle, prior to initiation of superovulation, may yield more consistent embryo production, perhaps due to a more tightly synchronized follicular wave emergence. Further, characterization of dynamics of this follicular wave may facilitate more consistent superovulation results and reduce costs.

Keywords: Bovine, superovulation, follicle, embryo

Effects of zinc on maturation and fertilization of bovine oocytes

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Zinc is an essential trace mineral in many species and is present throughout the body (highest concentrations in eye and prostate gland) having key roles in reproduction. ‘Zink spark’ (release of zinc) that occurs in oocyte following intracytoplasmic sperm injection or natural encounter with sperm influences embryo quality. In bovine oocyte, zinc is the most abundant transition element, with concentrations fluctuating occurring during maturation and fertilization events. Zinc has an important role in DNA stabilization during fertilization process. Objective was to determine the role of zinc in in vitro maturation and fertilization of bovine oocytes. We hypothesized that dose-dependent zinc supplementation would enhance oocyte maturation, whereas chelation of zinc would inhibit fertilization and early embryonic development. Bovine oocytes were obtained via follicular aspiration of postmortem ovaries harvested from an abattoir. Selected oocytes contained ≥ 3 layers of cumulus cells and a homogenous cytoplasm. Oocytes were separated into 4 maturation treatment groups, supplemented with 0, 5, 10, or 20 μM zinc. Supplementation doses were determined from analysis of zinc concentrations in adult cow plasma and follicular fluid (10.55 and 11.47 μM , respectively) and commercial maturation and fertilization medias (1.07 μM for each). Oocytes were considered mature if they had reached Metaphase II and had expelled their first polar body. Data were analyzed using Chi-Square. Rate of oocyte maturation was not different (78.1 ± 3.0 , 59.5 ± 4.3 , 69.8 ± 7.7 and $62.3 \pm 3.2\%$, respectively). Oocytes matured in 0 μM zinc, fertilized with frozen-thawed bull semen, were assigned to 2 groups. A zinc chelated group (2.7 mM of TPEN (tetrakis [2-pyridinylmethyl]-1-2-ethanediamine) in fertilization media) and a control group with no TPEN. Following fertilization, presumptive zygotes were independently cultured for 7 days (no TPEN). Data analyzed using ANOVA. TPEN-treated group had lower ($p < 0.05$) cleavage rate compared to control group (46.1 ± 2.3 and $75.6 \pm 3.4\%$, respectively). Embryo development rate was also lower ($p < 0.05$) in TPEN-treated group compared to control group (15.4 ± 0.03 and $37.8 \pm 0.03\%$). Average embryo developmental scores were lower ($p < 0.001$) in TPEN-treated group compared to controls (2.2 ± 0.1 and 3.4 ± 0.2). Zinc supplementation had minimal effects on in vitro maturation of oocytes; however, removing zinc during in vitro fertilization significantly decreased cleavage rate and embryo development.

Keywords: Bovine, zinc, fertilization, maturation, oocyte

Bull sperm morphology analysis varies greatly by reader

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Sperm morphology assessments are important for selection of a herd sire, with acceptable fertility influencing bovine industry economics. Studies have evaluated effects of reader experience and evaluation method on sperm morphology in other species, but apparently no study evaluated bulls. Semen was collected from 35 yearling bulls at a university bull test station and from each bull, 1 eosin-nigrosin morphology slide (with a monolayer of sperm) was created by a board-certified Theriogenologist. Seven individuals (blinded to bull IDs) assessed slides: 4 board-certified theriogenologists (DACT) and 3 fourth-year veterinary students (VS) who completed an advanced reproductive elective course. One-hundred cells from each slide were evaluated (2018 Society for Theriogenology classification) in oil immersion (100 x) objective utilizing a light microscope in 1 sitting. A second evaluation was completed by each individual ≥ 1 week later. Three DACT's performed additional evaluations of 200 and 400 sperm from first 5 slides to determine if assessing a greater number of sperm would increase agreement of morphologic characteristics within and between reviewers. Data were analyzed using separate mixed model analysis of variance (Proc GLIMMIX, SAS v9.4) to test fixed effects of reviewer type, reviewer, slide number or number of sperm read, and interactions. There was an interaction ($p < 0.0001$) of reviewer type and slide (VS versus DACT), indicating some slide ratings were very similar between reviewer types and some differed significantly. Among slides that differed, VS identified larger number ($p = 0.0001$) of morphologically normal sperm compared to DACT. In addition, there were differences ($p < 0.0001$) in coefficient of variation between reviewers, but no differences ($p = 0.78$) between reviewer type. Within DACTs, there were no effects ($p = 0.96$) of numbers read on percent normal sperm, indicating that evaluating additional sperm did not affect outcomes of BSE. There was an interaction ($p < 0.0001$) of number of sperm assessed and reviewer on time to complete and for some reviewers, there were significant increases in time to complete increased sperm assessment. Standard cutoffs for adequate morphology assessment utilized for decades are questionable. Bulls not classified as satisfactory potential breeders are possibly due to fewer number of sperm evaluated and variation between assessors. Further investigation into slide preparation, microscope, and reader experience is important to validate this method of evaluating bovine sperm morphology.

Keywords: Sperm, morphology, bull, assessment

Effects of motility activation and cryopreservation on fish sperm glycocalyx

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Mammalian sperm are coated with a glycocalyx that is formed during sperm development, maturation, and contact with seminal fluid. In contrast, little is known about sperm glycocalyx of fish sperm. Our objective was to describe fish sperm glycocalyx characteristics in different sperm types (testicular, stripped, and cryopreserved), motility activation statuses (inactive versus activated), and determine its role in fertilization using sauger (*Sander canadensis*) as a model. Our hypothesis was that glycocalyx characteristics would differ among sperm types, activation statuses, and be related to fertilization. Stripped, testicular, and cryopreserved sperm were prepared using our laboratory's published methodology for sauger.¹ All 3 sperm types were assessed in both inactive state (in extender ± DMSO) and activated state (1:20 dilution in hatchery water, 35 mOsm/kg). Three fluorescent lectins commonly used in mammalian sperm analysis (Wheat germ agglutinin [WGA], concanavalin A [ConA], and peanut agglutinin [PNA]) were used to evaluate presence and distribution of specific sugar moieties in sperm membrane (N – acetyl glucosamine [GlcNAc], α-mannose, and β-galactose, respectively) using flow cytometry and fluorescent microscopy. Fertilization was assessed in each sperm type and in stripped sperm pretreated with WGA prior to insemination. Linear mixed models were used to compare staining patterns (%) and fluorescent intensity (a.u.) among sperm types and activation statuses ($\alpha = 0.05$, $n = 10 - 12$). Glycocalyx of sauger sperm contained GlcNAc and α-mannose but lacked β-galactose moieties. WGA staining patterns differed among sperm types and with activation. Apical staining of WGA increased ~10 fold in activated sperm, except that frozen sperm had fewest cells with apical staining post-activation (46% testicular, 50 % stripped, and 5% frozen). A majority (> 90%) of sperm stained homogeneously with ConA independent of sperm type or activation status but fluorescent intensity was elevated (~ 2 - 3 fold) in frozen sperm compared to other sperm types. Activation did not affect fluorescent intensity of either WGA or ConA. Fertilization was similar for stripped ($79.3 \pm 4.2\%$) and testicular sperm ($64.0 \pm 5.7\%$); however, fertilization rates were significantly lower in both frozen sperm ($17.4 \pm 3.7\%$) and sperm pretreated with WGA ($14.0 \pm 1.4\%$). In conclusion, motility activation caused changes in glycocalyx of fish sperm. These changes, particularly in GlcNAc, were largely negated by cryopreservation, which could partially explain observed reduction in fertilization. Moreover, blocking GlcNAc moieties in sperm prior to insemination further demonstrated glycocalyx had a critical role in fertilization.

Keywords: Milt, sperm, cryopreservation, activation, glycocalyx

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Platelet-rich plasma reduces endometrial macrophages in postpartum beef heifers

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Postpartum endometritis in beef heifers can result in infertility and culling. Methods used by beef producers to treat endometritis can result in meat residues. Platelet-rich plasma (PRP) is an effective therapeutic treatment for endometritis in horses.¹ Therefore, we hypothesized that PRP would decrease endometritis in beef heifers. Objective was to compare endometrial responses of intrauterine PRP treatment to responses of platelet-poor plasma (PPP) or saline (SAL, 0.9% sodium chloride [pH = 5.0]) treatment in normal calving heifers. Fifteen normal calving crossbred beef heifers were randomly assigned to 3 intrauterine treatment groups. Heifers were examined at 14 and 28 days postpartum. Each examination included transrectal ultrasonographic measurements of cervical diameter, assessment of cervical discharge score (CDS) using a vaginal speculum, and quantitative aerobic bacterial culture and cytology of endometrial samples. After initial examination, heifers received 10 ml of either PRP, PPP or SAL via intrauterine infusion. PRP was prepared using routine methods previously described.² No treatment was administered after second examination. Cervical diameter, CDS, bacterial count, percentage of macrophages (%MAC), and percentage of neutrophils (%PMN) were compared between 2 time points using a Student's *t*-test. Groups were compared between time points using a repeated measures ANOVA, with a Tukey's post hoc test. Significance was defined as $p < 0.05$. Cervical diameter decreased in PRP and PPP groups, but not in SAL. CDS decreased in PRP and SAL groups, but not in PPP group. There was no difference in number of total aerobic bacteria count or %PMN among groups. However, %MAC decreased in PRP group, but not in PPP or SAL groups. PRP reduces in vitro responsiveness of bovine endometrial cells to bacterial endotoxins by downregulating several proinflammatory cytokines (e.g. interleukin-8).³ Research is needed to determine if PRP downregulates endometrial pro-inflammatory cytokines in vivo.

Keywords: Cervical discharge score, endometrial cytology, endometrial culture, endometritis, neutrophil, transrectal ultrasonography

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Ultrafast cooling reduced oxidative stress in vitrified bovine oocytes

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Cryopreservation of gametes has been a revolutionary tool in assisted reproductive technology field. Traditionally, cryopreservation was performed by slowcooling (0.2 - 10°C/minute) method which induces ice formation in extracellular space and dehydration of intracellular space. Recently, fastcooling or vitrification-based approaches replaced slow cooling in human and equine gamete cryopreservation, with higher cryosurvival rates and superior clinical outcomes. Obstacles for cells to survive fastcooling include intracellular ice formation and exposure to high concentrations of cryoprotectants (CPAs) required to achieve vitrification. Increasing cooling rates should reduce ice formation, and ice formation during warming can be reduced by reducing volume of media around cell. Cryotop system is industry leader, achieving cooling rates of -3,000°C per minute. NANUQ™ Hyperquenching Cryocooler, designed to maximize cooling rate for protein crystallography, combines automated fast (< 2 minutes) plunging with removal of cold gas layer above liquid nitrogen, leading to ultra-fast cooling rates of ~ 600,000°C per minute. We hypothesized that ultrafast cooling rates would reduce oxidative stress from cryodamage of vitrified/warmed bovine oocytes. Bovine oocytes were collected from abattoir-derived ovaries, matured in vitro, partially denuded of cumulus cells, then assigned to 1 of 4 groups: 1) negative control group which is not exposed to CPA and not cryocooled, 2) CPA control group which is exposed to CPA but not cooled, 3) Cryotop group with CPA exposure and cryocooling on Cryotops according to manufacturer protocols, and 4) NANUQ group with CPA exposure and cryocooling on microloops using NANUQ hyperquenching cooler. Oocytes were warmed, completely denuded and cultured for 1 hour in holding medium. Effect of treatment on oocyte oxidative stress was determined by fluorescent signal measured from cells after coincubation with green fluorescent general oxidative stress indicator CM-H2DCFDA. Images were transformed in ImageJ to derive corrected total cell fluorescence (CTCF) values, and these values were compared using ANOVA with Tukey multiple comparison on RStudio. A total of 281 oocytes were included. There were no differences ($p > 0.05$) in CTCF values between negative ($n = 77$) and vehicle control ($n = 93$) groups. CTCF values for cryocooled Cryotop ($n = 65$) and NANUQ ($n = 46$) groups were higher ($p < 0.001$) than control groups. For cryocooled groups, microloops cooled using NANUQ gave a lower ($p < 0.001$) CTCF value compared to Cryotop group. Experiment was repeated and in vitro fertilization was performed, but vitrified groups had poor cleavage rates and no blastocysts were produced, whereas negative and vehicle control groups had similar ($p > 0.05$) acceptable embryo development rates. NANUQ™ reduced postthaw oxidative stress of vitrified bovine oocytes compared to an industry standard vitrification device. Exposure to CPA did not have a significant effect on development.

Keywords: Automation, vitrification, in vitro fertilization, bovine

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Cooled-transported epididymides for donkey semen cryopreservation

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In the event of death or unforeseen castration for medical reasons, epididymal semen harvesting represents the last opportunity to preserve valuable jacks' genetics. Objective was to compare 2 semen cryopreservation methods (centrifugation versus noncentrifugation) on donkey cooled-shipped epididymides. We hypothesized that both methods would result in equivalent postthaw semen parameters. Experiment 1: donkeys (n = 7) housed at Bureau of Land Management in Tucson, AZ were surgically castrated. Testes and epididymides were placed in a plastic bag containing skim milk-based extender (25 ml, Botusemen, Botupharma, Scottsdale, AZ), and cooled-shipped overnight in an Equitainer to University of Illinois for semen cryopreservation. Each pair of epididymides was submitted to retrograde flushing with 5 - 10 ml with a cooling extender Botugold (Botupharma), or a similar volume of Botucurio (Botupharma). Botugold group samples were submitted to cushion-centrifugation (1000 g × 20 minutes), supernatant was discarded, and pellet was resuspended at 100 × 10⁶ sperm/ml in Botucurio. Botucurio group samples were resuspended to a similar final concentration. Semen from both groups was loaded in 0.5 ml straws, cooled at 5°C for 20 minutes, placed 5 cm over LN₂ for 15 minutes, and then plunged in LN₂ for storage. Experiment 2: donkeys (n = 20) housed at Peaceful Valley rescue in San Angelo, TX were surgically castrated and epididymides dissected away from testes and each pair was shipped similar to Experiment 1, except that a different shipping container was used (Botuflex, Botupharma). Each pair was submitted to retrograde flushing with either Botugold or Botucurio and processed exactly as in Experiment 1. For both experiments, 1 straw for each group was thawed at 38°C for 1 minute before sperm motility parameters assessments with CASA. Data were analyzed with R, using a mixed model Tukey's as post hoc. Motility parameters were affected by shipment method (p < 0.05) but not by cushion centrifugation (p > 0.05) processing (Table). Donkeys had poor postthaw sperm motility compared to what was expected for donkey ejaculates subjected to cryopreservation. This was apparently the first study involving epididymal semen in donkeys.

Table. Sperm motility parameters

		TM (%)	PM (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)
Experiment 1	Centrifugation	32.7 ± 5.2	23.0 ± 4.4	115.9 ± 3.3	45.1 ± 1.8	55.3 ± 0.9
	Noncentrifugation	26.0 ± 4.4	18.5 ± 4.9	109.5 ± 2.3	42.7 ± 2.6	52.7 ± 2.5
Experiment 2	Centrifugation	20.4 ± 2.0	11.0 ± 1.5	87.7 ± 3.3	33.9 ± 1.6	41.9 ± 0.9
	Noncentrifugation	26.0 ± 1.3	10.6 ± 1.3	88.9 ± 3.9	36.3 ± 1.7	44.9 ± 1.9

TM: Total motility; PM: progressive motility; VCL curvilinear velocity; VSL straight-line velocity; VAP: average path velocity.

Keywords: Epididymis, storage time, testis

Intrauterine infusion of platelet-rich or platelet-poor plasma to modulate persistent breeding induced endometritis in embryo donor mares

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Goal was to assess postbreeding endometrial inflammatory response and embryo recovery rates in mares susceptible to persistent breeding induced endometritis (PBIE) treated with platelet-rich plasma (PRP) and platelet-poor plasma (PPP). We hypothesized that administration of PRP and PPP mitigate endometrial inflammation in mares susceptible to PBIE and platelets have an additive ability to modulate this response. Mares (n = 12) that had 3 cycles were classified as susceptible to PBIE, based on a challenge with killed sperm and randomly assigned in a crossover design. Mares received intrauterine infusions of 40 ml of lactate Ringer's solution (LRS, control), or autologous PRP or PPP at 48 and 24 hours before and 6 and 24 hours postbreeding. PRP and PPP were prepared from blood, using a standard double centrifugation method. Platelet count and viability were assessed with spectral flow cytometry (CD41/61 coupled with IgG-PE conjugated). Ovulation was induced (≥ 35 mm follicle) with a gonadotropin releasing hormone agonist (histrelin) and mares were bred on subsequent day with fresh semen collected from a single stallion. At 6 hour postbreeding, uterine lavage (2 liters LRS) was performed immediately before infusion with treatment. Mares had daily measurements (height and width) of intrauterine fluid accumulation (IUF) at uterine body for 96 hours. Endometrial cytology was performed until 72 hours postovulation, and number of polymorphonuclear cells (# PMNs) was counted. Embryo flushing was performed 8 days postovulation with LRS. Recovered uterine fluid was aerobically cultured. Plasma was collected on the day of ovulation induction, 72 hours and 8 days postovulation to assess P₄ concentrations. Statistical analyses were performed with ANOVA-RM and posthoc Tukey's (IUF, # PMNs, P₄ concentrations), whereas fertility and number of positive bacterial cultures were assessed with multivariate regression. Mean platelet concentrations were 608.7 ± 62 and $47.5 \pm 12 \times 10^3/\mu\text{l}$ in PRP and PPP, respectively. In addition, there were no differences in platelet-viability between groups (97 ± 0.7 versus $97.2 \pm 0.6\%$). Both treatment groups significantly reduced the # PMNs on endometrial cytology from 24 - 72 hours postbreeding. Infusion of PRP and PPP resulted in a significant reduction in IUF postbreeding when compared to control. Estrous cycles assigned as control resulted in a higher percentage ($p < 0.05$) of positive cultures (42%), compared to PRP cycles (0%), whereas cycles treated with PPP were not different ($p > 0.05$; 17%) from other groups. P₄ concentrations substantially increased in both treatment groups on day 8 postovulation. Mares treated with PRP tended to have higher ($p = 0.08$) embryo recovery rates (83%) than mares in control group (42%), whereas PPP had intermediate embryo recovery (60%). In conclusion, plasma infusion can be used as an alternate method to modulate inflammatory response in mares susceptible to PBIE and PRP apparently had additional antimicrobial properties compared to plasma PPP.

Keywords: Embryo transfer, endometritis, reproduction, inflammation

Effect of pyruvate on lactate-induced spontaneous acrosome reaction in stallion sperm

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Stallion sperm metabolism is thought to be largely dependent on oxidative phosphorylation. Our recent study suggested that under “capacitating-like conditions” (presence of calcium, bovine serum albumin (BSA), and bicarbonate) only samples with pyruvate (as an energy substrate) had higher motility of stallion sperm. Presence of lactate alone was associated with substantial increases in spontaneous acrosome reaction rate in viable sperm, to almost half of all viable sperm at 4 hour incubation. However, motility was reduced in lactate treatment at 4 hours. We determined whether addition of pyruvate to a lactate-only-containing medium may increase stallion sperm motility while maintaining acrosome reaction rate in viable sperm. Fresh stallion ejaculates ($n = 9$) were washed and diluted to 30×10^6 sperm/ml using a Modified Whitten’s medium (MW) containing 7 mg/ml BSA and 10 mM lactate (Control), or 10 mM lactate with 0.5, 1, 5, or 10 mM added pyruvate. To control the effect of added substrate treatments, additional lactate was added at similar concentrations. Diluted sperm were incubated under a 5% CO₂ atmosphere for 4 hours. After incubation, samples were analyzed for total and progressive motility, %TMOT, and PMOT by CASA. Samples were stained with a fixable live/dead stain, followed by FITC-PSA and assessed for viability and acrosome status by flow cytometry. Data were rank-transformed prior to analysis by General Linear Model. The % TMOT was higher ($p < 0.05$) in 5 and 10 mM pyruvate (47 - 50%) than in lactate-only media (30 - 37%). Similarly, % PMOT was higher ($p < 0.05$) in 5 and 10 mM pyruvate (16%) than in lactate-only media (10 - 11 %). The % viable (55 - 57%) was not affected ($p > 0.05$) by any treatment tested. Addition of pyruvate was associated with a dose-dependent decrease in the proportion of viable sperm that were acrosome-reacted (AR; 42, 36, 31, 18, and 11% for 0, 0.5, 1, 5, or 10 mM added pyruvate), as compared to lactate (42, 40, 42, 39, and 40%, respectively). Results suggested that presence of pyruvate increased motility and decreased proportion of viable sperm undergoing spontaneous AR. Increases in motility and decreases in AR occurred at the same pyruvate concentration (5 mM added pyruvate). Increasing lactate concentration in the medium was not associated with an increase in AR. Findings may help to formulate media for stallion sperm capacitation, and highlighted the delicate energy balance required to support key functions of stallion sperm.

Keywords: Stallion sperm, acrosome reaction, energy substrate, pyruvate, lactate

***Streptococcus equi* subspecies *zooepidemicus* endometritis in mares:
culture, cytology, and antimicrobial susceptibility tests**

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Infectious endometritis is a significant cause of subfertility in broodmares, leading to devastating economic and emotional effects on horse owners and breeders each year. Aims of this observational, retrospective study were to report the percentage of positive uterine cultures and specific pathogens, evaluate the relationship between uterine culture and cytology results, and describe the results of antimicrobial susceptibility tests for *Streptococcus* isolates at an equine reproduction center in Colorado. We hypothesized that the most common bacterial pathogen would be *Streptococcus* sp. and that all *Streptococcus* isolates would be sensitive to beta-lactam antibiotics (i.e. penicillin G and ceftiofur), but not all isolates would be sensitive to other antibiotics. A double-guarded uterine swab and brush were used to collect 622 paired samples for culture and cytology, respectively, from mares at Colorado State University between 2017 and 2019. Swabs were plated onto Spectrum™ 4-Part (Colorado) plates (Vetlab Supply, Palmetto Bay, FL), which consisted of MacConkey agar, Tryptic Soy agar with 5% sheep blood, Chromogenic Gram-positive agar, and Chromogenic Gram-negative agar. Antimicrobial susceptibility tests were performed using the Kirby-Bauer disk diffusion method, incorporating amikacin, ceftiofur, ciprofloxacin, enrofloxacin, gentamicin, and penicillin G. Cytology slides were stained with a modified Wright-Giemsa stain (Astral Diagnostics Inc., West Deptford, NJ) and evaluated under 400 and 1000 x microscopy. A total of 513 cultures (82.5%) exhibited no growth of microbial pathogens, whereas 109 cultures (17.5%) had growth of 1 or more pathogens. Most common bacterial pathogens were *Streptococcus* spp. (73.4% of positive cultures), *Escherichia coli* (34.9%), *Klebsiella* spp. (5.5%), *Pseudomonas* spp. (3.7%), *Staphylococcus* spp. (0.9%), and *Enterococcus faecalis* (0.9%). A total of 20 positive cultures (18.3%) exhibited growth of more than 1 pathogen. A positive cytology was noted on 32.3% of samples collected from mares with a *Streptococcus* spp. culture and 42.1% of samples associated with an *Escherichia coli* culture. Antimicrobial susceptibility tests were performed on 38 isolates of *Streptococcus* spp. Percentages of susceptible isolates were as follows: amikacin (29.7%), ceftiofur (100%), ciprofloxacin (75%), enrofloxacin (61.1%), gentamicin (66.7%), and penicillin G (100%). In summary, *Streptococcus* spp. and *Escherichia coli* were the most common bacterial pathogens isolated. All *Streptococcus* isolates were susceptible to beta-lactam antibiotics. Use of antimicrobial agents to treat equine uterine infections should be based on antibiotic stewardship principles.

Keywords: Mare, uterine, culture, cytology, endometritis, antibiotics

Pregnancy rates in mares with pre and postovulation versus only postovulation frozen semen inseminations

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Mares may be bred by live cover or artificial insemination with fresh, cooled or frozen semen. Aim of this retrospective study was to compare pregnancy rates in mares after insemination with 2 doses of frozen-thawed semen (pre and postovulation) versus 1 dose (postovulation), in a commercial equine breeding program. Our hypothesis was that pregnancy rate will be similar. Reproductive records from mares bred with frozen semen at Colorado State University over a 3 year period (2017 - 2019) were evaluated retrospectively. Only first breeding cycle of the year was evaluated for any given mare. Mares were monitored by ultrasonography once daily when in estrus and administered 1 dose of 500 µg gonadotropin releasing hormone (GnRH) agonist (histrelin) at 8:00 pm when a dominant follicle of an appropriate size was present along with uterine edema. Mares were subsequently monitored 2 - 4 times daily to predict and subsequently confirm ovulation. Mares were bred by deep uterine horn insemination with either 2 doses of frozen-thawed semen, with 1 dose prior to anticipated ovulation plus a second dose after ovulation was detected, or only 1 dose of semen immediately after ovulation. Selection of the stallion, number of breeding doses allocated per cycle and number of straws per insemination were made by the owner of the mare and/or stallion. A mare was considered pregnant if an embryo was collected during a uterine lavage 8 days after ovulation, or if an embryonic vesicle was visible on 14 days postovulation via transrectal ultrasonography. Pregnancy rate was compared by Chi Square analysis. Records from a total of 156 estrous cycles were evaluated. Percentage of mares that ovulated within specific time frames after administration of histrelin were: ≤ 12 hours (8.3%), 13 - 24 hours (6.4%), 25 - 35 hours (2.6%), 36 - 40 hours (53.8%), 41 - 48 hours (19.2%), and > 48 hours (9.6%). Pregnancy rate of 54 mares inseminated prior to and after ovulation (46%) was not different ($p = 0.143$) from 102 mares inseminated with 1 dose of frozen and thawed semen immediately after ovulation (34%). There was also no difference in pregnancy rates based on mare age (3 - 10, 11 - 15 or 16 - 20 years) or reproductive status (maiden, barren, open or foaling). However, there was a difference ($p = 0.049$) in pregnancy rate between mares bred with semen of low progressive motility ($n = 8$; ≤ 15%) and high progressive motility ($n = 25$; > 60%). In summary, a numerical, but not statistical, difference in pregnancy rate was noted between mares bred with 2 doses of frozen semen (pre and postovulation) versus 1 dose of frozen semen, however, further studies are needed with more number in each group to confirm this finding. It is becoming increasingly common to breed mares with a single or partial dose of frozen semen, with veterinarians having little control over the quality and/or quantity of semen utilized.

Keywords: Equine, frozen semen, pregnancy, dose, motility

Use of serum amyloid A and other inflammatory markers to monitor inflammatory response in mares with periparturient complications

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Early recognition of excessive inflammation and infectious complications related to the peripartum period, leading to early institution of therapy, reduces postpartum discomfort and facilitates recovery. Because serum amyloid A (SAA) is a highly sensitive marker of inflammation, measurements of SAA and other inflammatory markers in postpartum mares may be valuable in assisting clinical assessment of periparturient complications. We hypothesized that mares with peripartum complications substantially altered inflammatory responses compared to normal postpartum mares. Aims were to: 1) determine if inflammatory markers (serum amyloid A (SAA), fibrinogen, white blood cell count (WBC), and iron) are affected by normal parturition; and 2) investigate if parturition-related complications affect concentrations of WBC, SAA, fibrinogen, and iron. A retrospective case-control study included 118 postpartum mares, 72 clinically healthy (CH) mares accompanying sick foals and 46 mares with periparturient complications (PC) admitted to the University of Copenhagen Large Animal Teaching Hospital from 2008 - 2017. Periparturient complications were divided into 3 groups: metritis ($n = 9$), dystocia ($n = 13$) and others ($n = 24$). A multivariate linear regression analysis evaluated the effects of health status of mare (CH or PC), time after foaling (Day PP) and individual mare on blood parameters. Independent-samples Student's *t* test analyzed differences in inflammatory parameters between CH and PC mares at different days after foaling. CH mares had SAA, WBC, and iron concentrations within reference intervals for first week postpartum (PP). Mean fibrinogen concentrations increased above upper reference limit in both CH and PC mares during the first week PP, but PC mares had higher ($p < 0.05$) concentrations compared to CH mares. Health status of mares had substantial influence on concentrations of SAA ($p < 0.0001$), fibrinogen ($p < 0.0001$), and iron ($p = 0.009$), and day PP had an effect ($p = 0.02$) on WBC in both CH and PC mares. Fibrinogen concentrations increased ($p < 0.05$) on days 2, 3, and 7 and SAA concentrations on days 1 - 7 ($p < 0.05$), and WBC and iron concentrations decreased ($p < 0.05$) on days 1 - 3 ($p < 0.05$) in PC mares compared to CH mares. Iron and WBC concentrations were, however, within reference concentrations, for both groups of mares. Mares diagnosed with metritis had lower ($p = 0.008$) iron concentrations compared to mares with other periparturient complications. Inflammatory markers SAA and WBC were not affected by normal parturition and can be used to monitor inflammation and infection in mares with peripartum complications. Healthy postpartum mares had increased fibrinogen concentrations within first 7 days after parturition. Periparturient complications elicited, however, substantial higher fibrinogen concentrations compared to concentrations in normal postpartum mare. A major fibrinogen response, therefore, still indicates periparturient complications in a postpartum mare, and can, together with SAA and WBC, be used to monitor the inflammatory response related to periparturient complications.

Keywords: Acute phase response, serum amyloid A, periparturient complications, postpartum

Clinical effects of prebreeding intrauterine platelet-rich plasma in mare

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Platelet-rich plasma (PRP) is being used with increasing frequency in both human and veterinary medicine. By concentrating platelets, growth factors are recruited to aid in tissue healing and repair. In women, intrauterine PRP treatment improved endometrial receptivity. In mares, a limited number of studies focused on treating subfertile mares with intrauterine autologous blood products. Our aim was to examine the safety and efficacy of intrauterine administration of autologous PRP prepared with a commercial platelet isolation device (Restigen PRP[®], [platelet buffy coat concentration device], Owl Manor, Warsaw, IN). We hypothesized that the treatment would be both safe and effective in addressing intrauterine inflammation, based on the frequency of adverse events, intrauterine fluid grade and pregnancy rate following treatment. Notably, we elected to focus on clinical end points rather than histologic or cytologic evaluations, and used frozen-thawed semen as opposed to fresh or cooled transported semen. In this clinical-based crossover study, inclusion criteria were failure to achieve pregnancy after artificial insemination with frozen semen, absence of clinical evidence of infectious endometritis, and normal physical exam parameters. Eighteen mares of various breeds, ages and parities were used. Each mare served as her own control and was bred to the same stallion as the previous cycle. Breeding management was performed in a routine manner. Autologous PRP was prepared by an experienced operator using Restigen PRP kits and uterine body infusion was performed 12 - 48 hours prior to artificial insemination. Postinsemination, intrauterine fluid was graded via transrectal ultrasonography using a predetermined scale based on volume and echogenicity. Pregnancy status was determined via transrectal ultrasonography from 13 - 16 days postovulation or via embryo flush performed 7 - 8 days postovulation. No adverse events were recorded in association with intrauterine infusion of PRP. Data were analyzed using nonparametric tests. There was no difference ($p > 0.05$) in post-breeding intrauterine fluid score between treatment and control groups. Postbreeding intrauterine fluid score improved in 7/18 mares (39%) and was unchanged in 6/18 (33%). There was substantial effect of treatment on pregnancy rate, as 11/18 mares (61%) became pregnant in the treated cycle as opposed to 0/18 (0%) in control cycle. Results indicated that intrauterine infusion of autologous PRP prepared with a commercial platelet isolation device in the periovulatory period was a safe procedure, and associated with improved breeding outcomes in this population of mares. To better determine efficacy, further studies are in progress to differentiate these results from those of second cycle pregnancy rates without PRP use.

Keywords: Mare, platelet-rich plasma, frozen semen, artificial insemination, safety, inflammation

Expression of prostaglandin E₂ and oxytocin receptors in stallion accessory sex glands

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It is unknown whether oxytocin and prostaglandin receptors are present in accessory sex glands. Furthermore, if receptors are present, their importance and distribution is unclear. Knowledge of normal receptor expression in intact and castrated males may help to improve semen collections in difficult stallions and may further our understanding of accessory sex gland physiology. Objectives were to characterize expression of prostaglandin E₂ (EP2, EP4) and oxytocin (OXTR) receptors' genes in equine accessory sex glands using immunohistochemistry (IHC) and to determine if localization varied based on age or reproductive status. We hypothesized that EP2, EP4, and OXTR are more strongly expressed in mature stallion glands than in gelding or fetal glands. At euthanasia, ampulla, prostate, vesicular, and bulbourethral gland tissue were collected from mature stallions (> 5 years old, n = 3), mature geldings (> 5 years old, n = 3), and male fetuses (280 days of pregnancy, n = 3). Fresh tissues were fixed in 10% neutral buffered formalin, then embedded in paraffin until processing. Tissues were sectioned in 5 µm sections and stained with rabbit antihuman polyclonal antibody for EP2 or EP4 (Santa Cruz Biotechnology), or mouse antihuman monoclonal antibody for OXTR. Slides were processed using an IHC Select HRP/DAB kit (Millipore, Burlington, MA). Protein localization of EP2, EP4, and OXTR was evaluated by IHC, and staining was characterized as absent, mild, moderate, or strong. Prostaglandin E₂ and oxytocin receptors' genes were expressed in all glands. EP2 expression was mild to moderate in luminal epithelium of all glands. EP4 was strongly expressed in luminal epithelium of all glands, moderately expressed in smooth muscle of ampulla and prostate, and mildly expressed in submucosa of vesicular gland. Presence of EP2 in lumen should not come as a surprise, since PGE₂/EP2 play an important role in secretory epithelium in other organs (e.g. lungs and stomach). Interestingly, upregulation of EP2/EP4 are associated with tumor development in accessory glands of humans and rodents and other tissues; however, neoplasia in accessory sex glands of stallions is extremely rare. EP2 and EP4 play an important role in contraction and relaxation in the esophagus and it is likely they play a similar role in the stallion accessory sex glands. Moderate expression of OXTR in epithelium and mild expression in glandular stroma, and its receptor presence suggested that oxytocin may help regulate glandular secretions. Presence in equine fetus suggested that these receptors may play a role in development of accessory sex glands. Lack of apparent variation in these receptors in castrated horses suggested a steroid-independent role for receptor expression in glands. In conclusion, expression of EP2, EP4, and OXTR was confirmed in all male equine accessory sex glands, including equine fetus. Relative roles of these receptors in stallion accessory sex glands physiology, during ejaculation, and disease remain to be studied.

Keywords: Prostate, horse, ampulla, seminal vesicle

Fungal endometritis caused embryo loss in a maiden mare

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A 6 year old, maiden, Standardbred mare was inseminated with chilled, extended semen (from same stallion) during 2 consecutive estrous cycles. Following first cycle insemination, traces (< 1 cm diameter) of fluid were occasionally detected in uterine body during routine transrectal ultrasonographic examination. Day after second cycle insemination, transrectal ultrasonographic examination confirmed ovulation and absence of uterine fluid. Thirteen days postovulation, she was reexamined for an early pregnancy diagnosis. Embryonic vesicle (9 mm) was detected in uterine body with no free fluid in uterus. At scheduled midpregnancy diagnosis (28 days postovulation), ~ 10 cm diameter, mainly hypoechogenic, fluid pocket with mixed echogenic contents was detected in uterine body. No uterine edema was detected and cervix was closed. Uterine lumen was initially lavaged with sterile saline, followed by 1 liter of sterile saline with 20 ml of white vinegar. Reflux from saline uterine lavage was turbid and included debris and round, “fluffy” white objects 1 - 3 mm in diameter. Samples from lavage were sent for laboratory identification. A smear was stained with ‘Diffquick’ and assessed in the examining area. Debris, epithelial and inflammatory cells, stout rod-shaped bacteria, and nonfruiting fungal hyphae were detected. Multiple drug resistant *Enterobacter aerogenes* was identified in culture. A fungus was cultured, but not definitively typed, although it was suspected to be *Acremonium* spp. Mare was treated with prostaglandin F_{2α} analogue (125 µg) and oxytocin (10 IU) IM twice daily for 2 days, and reexamined 4 days later. Minimal (< 0.5 cm diameter) intrauterine fluid was detected. A clitoral swab was collected for fungal isolation, but was unrewarding. A double-guarded uterine swab was collected prior to next insemination, but culture results were unrewarding. She was inseminated again 26 and 58 days after diagnosis of fungal endometritis. She was diagnosed pregnant 14 days following second insemination. Mare had good perineal conformation with no previous history of intrauterine antibiotics or immunomodulating pharmacological (corticosteroids or NSAID’s) agents (considered risk factors for fungal endometritis). Putative fungus, *Acremonium* spp. is typically considered to be a plant associated symbiont or pathogen. Fungal endometritis is considered most likely reason for embryonic loss in this mare. To the authors’ knowledge, there are no reports of fungal endometritis as a likely cause of embryo loss (prior to 40 days) in mare.

Keywords: Mare, fungal endometritis, embryo, embryonic loss

Pyometra associated with hyperammonemia in a mare

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Pyometra is an abnormal accumulation of purulent debris in uterine lumen. This accumulation may be due to cervical adhesions that interfere with drainage of uterine fluids. However, accumulation can also occur without cervical obstruction. In mares, clinical signs of systemic disease are rarely present. Etiology of pyometra in mares remains to be fully determined. A 20 year old multiparous Paint mare was presented for inappetence and lethargy. On physical examination, her mentation was obtunded with a low head carriage, head pressing, and facial grimace. She was underconditioned (BCS 3/9) with a distended abdomen. Her vital parameters were unremarkable except decreased (97.1°F) temperature. On abdominal palpation per rectum, a mass filling entire abdominal space was palpated which was confirmed to be uterus on ultrasonographic examination. Flocculent hyperechoic fluid (31 cm) was visualized. A complete blood cell count showed neutrophilia with toxic left shift, hyperfibrinogenemia and thrombocytosis. Chemistry revealed a metabolic acidosis associated with a high anion gap, mild azotemia, hyperglobulinemia, hypoalbuminemia and moderately elevated ALP, GGT, mildly elevated SDH, hypertriglyceridemia and hyperammonemia (300 µg/dl). Uterine drainage and lavage were performed. Thick, pale brown, and malodorous fluid (101 liters) was recovered from uterus. Uterine fluid sample was submitted for culture, which yielded *Escherichia coli* and anaerobes (*Fusobacterium necrophorum*, *Prevotella* sp, *Bacteroides* sp, *Fusobacterium nucleatum*). Mare received antimicrobials for a total of 27 days, including initial gentamicin (6.6 mg/kg IV once daily) and penicillin G procaine (22,000 IU/kg IM twice daily) for 3 days, followed by trimethoprim sulfamethoxazole (30 mg/kg PO twice daily) on Day 4 due to clinical improvement. Metronidazole (20 mg/kg per rectum every 8 hours) was initiated on Day 15. Flunixin meglumine 0.3 mg/kg twice daily was also administered for 3 days, and 5 IU of oxytocin was administered IM every 3 hours for 2 treatments. Uterus was lavaged daily for first 3 days, followed by every other day for remainder of hospitalization. Cervix developed adhesions, endometrium remained thickened, and fluid continued to accumulate between lavages (6 - 8 liters per lavage). A cervical wedge resection performed on day 27 of hospitalization resulted in full thickness vaginal tear. Mare was humanely euthanized. Clinical presentation of pyometra concurrent with signs of systemic disease observed in this mare provided new information regarding physiopathology of pyometra.

Keywords: Uterine infection, hyperammonemia, mare pyometra

Diagnosis of omphalocele in a Toggenburg goat fetus during pregnancy

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Multiple ovulation embryo transfer was performed on a 5 year old Toggenburg donor doe. Washed and trypsin-treated fresh embryos were transferred into 4 primiparous Toggenburg does. Pregnancy was confirmed via transabdominal ultrasonography at day 33 of pregnancy. Does were regularly monitored throughout pregnancy. A doe that received 3 embryos, lost 1 in embryo and 1 fetal (male fetus) stages, respectively, and gave birth to 1 live kid. At day 61, transabdominal ultrasonography of this male fetus identified an umbilical abnormality that was monitored until its death at day 104 of pregnancy. This male fetus was delivered as a mummified fetus with its unaffected, full term twin at day 149 of pregnancy. Omphalocele was diagnosed in this male fetus, based on ultrasonographic findings of a ventral abdominal wall defect with herniation of abdominal viscera into umbilical cord base. At parturition, an abdominal wall defect was apparent, but omphalocele was no longer observable, due to fetal autolysis. Significant postmortem findings included a multivessel umbilical cord, concurrent mummification and maceration of affected fetus, long bone abnormalities, and cleft palate. Infectious causes of caprine abortion were ruled out. This case demonstrates practitioner's ability to diagnose umbilical defects, including omphalocele, using transabdominal ultrasonography during routine pregnancy diagnosis. Transabdominal ultrasonography can identify omphalocele's bilaminar sac presence to distinguish omphalocele from gastroschisis, which has similar appearance. This sac may tear prenatally or during delivery, making perinatal differentiation of these 2 conditions more challenging.

Keywords: Omphalocele, mummification, maceration, pregnancy, umbilicus

Presence of *Tritrichomonas foetus* in a chronically infected bull's urethra

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A 5 year old Maine Anjou Angus cross bull was donated to Auburn University College of Veterinary Medicine. Bull's smegma was collected via preputial scraping with an artificial insemination pipette attached to a 20 ml syringe. Smegma sample was placed into a vial with Modified Diamond's media and submitted to Thompson Bishop Sparks Alabama state diagnostic laboratory (TBSASDL) for testing for *Tritrichomonas foetus* (*T. foetus*). Bull was positive for *T. foetus* DNA, based on real time polymerase chain reaction (RTPCR). Bull was tested for *T. foetus* on numerous occasions annually over a period of 5 years and was deemed chronically infected with *T. foetus*. Prior to euthanasia, smegma was collected and placed in Modified Diamond's media. Bull and its smegma sample were submitted to TBSASDL for necropsy and RTPCR, respectively. Reproductive tract was dissected free; 1 x 1 cm sections were cut from prostate, right and left ampullae, right and left vesicular glands, and sections of urethra. The urethra was sectioned at specified locations; 5 cm proximal from distal end of urethra; 23 cm proximal from distal end of urethra; 5 cm distal to last bend of sigmoid; and 13 cm proximal to sigmoid and urethra at ischium level. All samples were placed in Modified Diamond's media and submitted to TBSASDL for culture and testing for *T. foetus* DNA via RTPCR. Reproductive tract was sectioned from most proximal portion to most distal portion. Gloves were changed between each collection of tissue sectioned. Instruments were cleaned following collection of each tissue section to prevent accidental contamination. A new sterile blade was used when sectioning each portion of reproductive tract. Prostate, vesicular glands, ampullae, and proximal urethra were all negative for *T. foetus* DNA. Smegma from preputial scraping and urethral sections 5 cm proximal from distal end of urethra, and 23 cm proximal from distal end of urethra were positive for *T. foetus* DNA via RTPCR with a cycle threshold (CT) of 30, 34.3, and 36, respectively. CT values from 30 - 37 are considered a positive reaction, indicative of moderate amounts of target nucleic acid present in this case. This is first documentation of *T. foetus* in a more proximal location in urethra. A few studies identified *T. foetus* presence in distal urethra of some bulls, but not in every bull. However, those studies were performed prior to availability of PCR; therefore, there might have been some false positives from bulls tested. This case provided important information for researchers assessing clearance of *T. foetus* from infected bulls, as topical treatments alone will not be curative to all bulls.

Keywords: Cattle, trichomonias, *Tritrichomonias foetus*, urethra

Hypertestosteronism in an intact female alpaca secondary to an interstitial cell tumor

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An apparently healthy, 10 year old female alpaca was presented for infertility and male-like behavior. She had failed to conceive after exposure to intact males (multiple observed breeding) since her last parturition. All female alpacas housed with her were determined to be pregnant. She had been regularly mounting other female alpacas for over 1 year. A month prior to presentation, she had elevated serum testosterone concentrations. Abdominal computed topography (CT) identified a soft tissue (attenuating mass like structure with multiple cystic areas) associated with right uterine horn and right ovary, consistent with a neoplastic process. Bilateral ovariectomy was performed; left ovary was 6 cm in length and had 2 firm nodules (0.9 and 0.8 cm in diameter) and cystic areas were identified in right ovary. Both ovaries were submitted for histopathologic evaluation. Pre and postop blood samples were submitted to endocrine laboratory (University of California, Davis, Davis, CA) for inhibin B, progesterone, and testosterone. Preop blood samples had following concentrations; inhibin β_B : 40.1 pg/ml; progesterone: 1.2 ng/ml; and testosterone 1538.3 pg/ml; and postop blood samples had: inhibin β_B : 4.9 pg/ml; progesterone: 0.11 ng/ml; and testosterone: 26.4 pg/ml. Blood samples submitted from a healthy intact female alpaca (to provide a reference range for normal values) had following concentrations; inhibin β_B : 41.7 pg/ml; progesterone: 0.01 ng/ml; and testosterone: 33.7 pg/ml. Preop hormone concentrations confirmed hypertestosteronemia and its ovarian source was confirmed by postoperation hormone concentrations. Lowering of inhibin β_B and progesterone in postop sample was consistent with expected changes that occur following removal of ovaries. Right ovary had multiple cysts lined with attenuated to ciliated cuboidal eosinophilic material and a diagnosis of cystic rete ovarii, whereas left ovary had 2 neoplastic structures composed of packed polygonal cells with indistinct borders and abundant eosinophilic cytoplasm. No mitotic figures observed (10 fields; 400 x magnification) indicating a benign nature of the tumor (interstitial cell tumor). Testosterone produced by left ovary was most likely responsible for cystic condition of right ovary and cystic rete ovarii. Interstitial or Leydig stromal cell tumors are rare ovarian tumors that belong to sex cord stromal tumors group. These tumors account for < 0.1% of all ovarian tumors in women and are even more rare in camelids. Interstitial cell tumors are characteristically benign and unilateral and produce testosterone. Although interstitial tumors are considered benign neoplastic conditions, they have significant effects on fertility, often ending reproductive life, as in this case. It is important to determine hormone concentrations to diagnose hypertestosteronemiam, followed by histopathology of ovaries in females exhibiting male-like behavior and virilization, to assist in definitive identification of interstitial cell tumors.

Keywords: Camelid, ovary, interstitial cell tumor, Leydig cell, hypertestosteronemia

Hemicastration for a suspected spermatic cord torsion in a dog

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A 4 year old domestic Black Mouth Cur dog, 32.5 kg, was evaluated for infertility. The dog had sired, with natural mating, 3 litters. Approximately 9 months prior, dog was reportedly febrile (40 - 41 °C) and had a stiff gait. Leptospirosis was suspected, based upon a pet-side antibody detection test. Veterinary care included subcutaneous fluid therapy and 3 week course of doxycycline. Two months prior to presentation, a bitch to which the dog was mated did not achieve pregnancy and upon subsequent recent semen collection, oligospermia and asthenozoospermia were evident. On initial presentation, at physical examination, dog appeared clinically systemically healthy, but scrotal content was abnormal. The left testis was normal in size and texture, whereas the right testis was firm and enlarged. Both cauda epididymides felt slightly firm and both testes apparently had normal orientation. Scrotal skin was normal. Prostate felt normal on transrectal examination and was nonpainful. On transcutaneous ultrasonographic examination, prostate had normal parenchyma, whereas right testis was abnormal, with mixed echogenicity. Blood flow was evident in both spermatic cords. Inhouse serum *Brucella canis* rapid slide agglutination test (RSAT) was positive and 2 mercaptoethanol-RSAT was negative, whereas New York State Diagnostic Laboratory agar gel immunodiffusion II and SAT results were negative. Semen had 120 million sperm with asthenozoospermia (< 10% total motile) and teratozoospermia (24% normal). Hemicastration of the right testis was recommended and performed 6 weeks later. Absence of testicular parenchyma (consistent with tissue necrosis) and structurally normal epididymis devoid of sperm were observed on histopathology. Prior spermatic cord torsion as the etiology of initial illness with subsequent complete testicular degeneration was postulated. Approximately 3 months postsurgery, an ejaculate contained 110 million sperm and showed significant improvement (50% progressively motile and 60% morphologically normal sperm). Determination of precise ovulation timing of bitch was recommended for future breeding to maximize chance of pregnancy. Hemicastration and reexamination at appropriate interval in management of unilateral testicular disease are necessary, as demonstrated by this case.

Keywords: Canine, infertility, testicular degeneration, spermatic cord torsion, hemicastration

Repair of a full thickness uterine tear via iatrogenic uterine prolapse in an anesthetized Thoroughbred broodmare

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A 9 year old multiparous Thoroughbred mare at 347 days of pregnancy was presented to Rhinebeck Equine, LLP for a delay in progression of stage II parturition. Farm foaling attendant reported that the foal was not present in vaginal vault and instead 2 feet and a nose were present within rectum. Mare was transported to clinic immediately. On presentation, there was no evidence of a full thickness rectovaginal tear; however, the foal was displacing rectal mucosa dorsally. Mare was induced under general anesthesia and a live foal was delivered via controlled vaginal delivery. Following recovery of anesthesia, mare was assessed, based on farm history. There was no evidence of a rectovaginal fistula. Transabdominal ultrasonography revealed presence of swirling echogenic free fluid. Abdominocentesis was performed and hemorrhagic, nonclotting fluid obtained. Fluid analysis revealed elevated total nucleated cell count ($7.55 \times 10^3/\mu\text{l}$ [reference range $1.5 - 5 \times 10^3$ cells/ μl]), elevated abdominal lactate (3.8 mmol/l [reference range 0 - 1.5 mmol/l]), and elevated total protein (2.6 g/dl [reference range < 2 g/dl]). Perineum was aseptically prepared. Digital manual transvaginal examination confirmed an ~ 15 - 20 cm full-thickness uterine tear on dorsal aspect of uterine body, to right of midline. Mare was induced under general anesthesia for uterine laceration correction. A ventral midline celiotomy was attempted, but due to caudodorsal location and size of the defect, surgical repair was not achieved. While in dorsal recumbency, uterus was manually prolapsed through vagina by placing gentle traction on attached fetal membranes. This approach allowed visualization and hand-sewn repair of laceration. The Dutch method (umbilical vessel water infusion) was used to facilitate removal of fetal membranes prior to uterus replacement. Recovery was uneventful. Rebreeding was delayed until the following season. Mare conceived on the second cycle and delivered a live normal colt at day 356 postovulation. To authors' knowledge, iatrogenic uterine prolapse and umbilical vessel water infusion have not been reported. This technique is used by authors with good outcomes in mares with large, miduterine body laceration.

Keywords: Mare, dystocia, uterine tear

Therapy and evaluation of early embryonic loss in a subfertile bitch

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A five year old intact female Cardigan Corgi was presented with a history of infertility and pregnancy loss. Interestrus interval reported was consistent (every 6 - 7 months), except for the last interval, which was reduced to 4½ months. Bitch was inseminated 5 times in 2 years. Surgical insemination using frozen semen was successful, and 2 puppies were whelped. Since then, bitch was inseminated with fresh or cooled semen 4 times (1 vaginal, 1 surgical, and 2 transcervical). It was suspected that pregnancy was either not established or lost in midpregnancy. Last insemination was performed 2 months before presentation. Local veterinarian diagnosed pregnancy via a relaxin assay and abdominal ultrasonography. Due to history of infertility, another abdominal ultrasonography was performed the following week, and early embryonic death was noted. On presentation, bitch appeared healthy and bright with normal physical parameters, and there was no vulvar discharge. Complete blood count and chemistry were within normal limits. Vaginal cytology had scant cellularity; noncornified parabasal cells, scant red blood cells, with few neutrophils and bacteria noted. Serum progesterone concentrations were 1.1 ng/ml. *Brucella canis* serology was negative. Vaginal samples were submitted for Mycoplasma and aerobic cultures. Scant growth was noted on Mycoplasma culture. Ultrasonographic evaluation revealed the presence of 8 mm echogenic fluid in right uterine horn and 2 mm fluid in left uterine horn. Both uterine horns contained thickened foci with a hypoechoic central region and hyperechoic luminal margins, consistent with necrotic placental zones. Uterus had multifocal cystic endometrial hyperplasia with several 4 mm cysts. Ovaries were normal and had luteal tissue. Renal cortical calcifications with normal abdominal content were noted. As bitch appeared clinically and systemically stable, 3 weeks of oral enrofloxacin (12 mg/kg) and Clavamox® (14 mg/kg) were prescribed for possible risk of fulminant pyometra. Two weeks of topical vaginal Misoprostol, 1 µg daily, was prescribed for cervical dilation. Future mibolerone administration was discussed to prolong anestrus, but was declined. Electro-acupuncture was performed several times and bitch was given an oral "lotus formula". One month later, ultrasonographic examination revealed an involuted uterus with no free fluid present and 2 small cysts. Two months later, no free fluid or cysts were present. Bitch will be bred at next estrus to a proven male, with results pending.

Keywords: Canine, infertility, cystic endometrial hyperplasia, early embryonic death

Generation of hormone-responsive organoids from fresh and cryopreserved equine endometrium: comparison between domestic and endangered Przewalski's mares

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Endometrium is responsive to signals associated with reproductive cyclicity and pregnancy. Organoids, 3-dimensional cultured cell structures, mimic in vivo tissue structure and function better than other cell culture methods and may improve understanding of endometrial physiology. However, there are no reports of endometrial organoids generated from equine tissues. Our hypothesis was hormonally-responsive organoids can be generated from fresh and cryopreserved endometrium from domestic (*Equus ferus caballus*) and Przewalski's (*E. f. przewalskii*) mares. Our objective was to compare histology, immunohistochemistry (IHC), transmission electron microscopy (TEM), and gene expression in organoids derived from fresh and frozen-thawed domestic horse and frozen-thawed Przewalski's horse endometrial tissues. Domestic (n = 11) and Przewalski's (n = 3) mare endometrial biopsies were collected, bisected, and either cryopreserved in 10% DMSO or dissociated enzymatically. Endometrial gland fragments were embedded in Matrigel and overlaid with DMEM/F12 (cell culture) plus supplements. Organoids (8 wells for each treatment) were cultured through 2 passages (6 days/passage) and then, for the third passage, incubated with no hormones (Control; C), or supplemented with: 1) 1 μ M progesterone (P₄) for 6 days; 2) 10 nM estradiol-17 β (E₂) for 6 days; 3) E₂ for 2 days then P₄ for 4 days; 4) C for 5 days then 10⁻⁵M oxytocin (OT) for 24 hours; or 5) C for 5 days then 10⁻⁶M OT for 24 hours. At the end of culture, organoids were analyzed for histology, TEM, IHC, and gene expression. Gene expression was analyzed (R project) and Kruskal-Wallis and Dunn were used as posthocs. Organoids were derived from fresh and frozen-thawed domestic mare and frozen-thawed Przewalski's mare endometrium with histology and TEM revealing cystic structures of epithelial cells with microvilli and intact secretory apparatus. Expression (IHC) of estrogen receptor- α (ER α) and progesterone receptor (PR) was restricted to the nuclei and prostaglandin endoperoxide synthase-2 (PTGS2) to the cytoplasm of endometrial cells. Expression of ER α and oxytocin receptor (OXTR) decreased (p < 0.05) while PR and prostaglandin E synthase (PGES) increased (p < 0.05) in fresh-derived organoids exposed to certain treatments compared to C. ER α , E-cadherin, and PTGS2 expression decreased (p < 0.05) and PGES increased (p < 0.05) in cryopreserved-derived organoids exposed to selected treatments compared to corresponding C. Subspecies comparison revealed an increase (p < 0.05) in OXTR expression in the Przewalski's horse. This is the first report of equine endometrial organoid generation and long-term culture. This novel method of in vitro equine endometrial culture may lead to development of improved in vitro evaluation of normal reproductive physiology, pathological conditions, and potential therapies for uterine diseases in both domestic and endangered equids.

Keywords: Endometrium, organoid, mare, Przewalski's, in vitro

Semen parameters and fertility of cooled stallion semen extended with sodium caseinate and phosphocaseinate based extenders

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Aim was to compare 2 commercially available equine semen extenders based on phosphocaseinate (INRA 96 IMV, US) and sodium caseinate associated with cholesterol-cyclodextrin (Botugold, Botupharma, US), on sperm parameters and fertility of stallion semen after cooling. We hypothesized that use of a sodium caseinate cholesterol-enriched extender and seminal plasma removal would result in superior parameters in cooled stallion semen. In Experiment 1, 45 ejaculates collected from 9 mature stallions were extended to 50×10^6 /ml (SP group) with INRA96 or Botugold and processed through cushion centrifugation (CC group) ($1000 \text{ g} \times 20$ minutes) before resuspension at 100×10^6 /ml with respective extender. Noncentrifuged and CC samples were placed in 3 (Equitainer, Botuflex, and Equine-Express II) passive cooling devices for 24 or 48 hours. Seminal parameters assessed at 0, 24, and 48 hours, included total motility (TM), progressive motility (PM) with CASA (Spermvision, MOFA, Verona, WI), and membrane integrity and mitochondrial membrane potential with spectral flow cytometry (Zombie Green and Mitotracker Deep Red). In experiment 2, mares ($n = 12 \times 2$ estrous cycles) were bred with 1 billion total sperm from 1 fertile stallion, 40 hours postinduction of ovulation with gonadotrophin releasing hormone agonist, histrelin. Semen was collected, extended in INRA96 and Botugold, and stored for 48 hours in Equitainer, and mares were randomly bred with 1 extender in an alternate order. Mares had transcervical embryo flushing performed on day 8 postovulation. Data were analyzed using mixed models (R project) and Tukey's as posthoc. Addition of cholesterol to sodium caseinate extender, resulted in superior ($p < 0.05$) semen parameters. There were no differences between container type. Cushion centrifugation increased ($p < 0.05$) sperm kinetics parameters and mitochondrial membrane potential and not ($p > 0.05$) plasma membrane integrity (Table). In Experiment 2, embryo recovery rates were identical ($p > 0.05$) between extenders (50%). In conclusion, we inferred that novel extender (Botugold) is a suitable commercially available product that can be used for stallion semen cooling with different containers, with superior sperm parameters than traditional INRA96 and at least with comparable fertility.

Table. Effect of cushion centrifugation on semen quality parameters

Time in hours		Total motility (%)		Progressive motility (%)		Sperm with intact plasma membrane (%)		Sperm with high mitochondrial membrane potential (%)	
		INR96	Botugold	INR96	Botugold	INRA96	Botugold	INRA96	Botugold
24	SP	70 ± 1^{aw}	75 ± 1^{bw}	63 ± 1^{aw}	67 ± 2^{bw}	72 ± 3.7^a	76 ± 2.3^b	23 ± 3^w	28 ± 2^w
	CC	74 ± 1^{ax}	76 ± 1.3^{bx}	67 ± 1^{ax}	69 ± 1^{bx}	68.4 ± 2.8	72 ± 2.2	28 ± 2^x	34 ± 2^x
48	SP	56 ± 2^{aw}	66.2 ± 1.8^{bw}	50 ± 2^{aw}	58 ± 2^{bw}	66 ± 2.2^a	69 ± 1.7^b	71 ± 3.3^w	71 ± 3^w
	CC	70 ± 2^{ax}	68.9 ± 1.6^{bx}	63 ± 1.8^{ax}	65 ± 2^{bx}	65 ± 2.3	66 ± 2.0	76 ± 2.7^w	76 ± 3^w

SP: before centrifugation, CC: after centrifugation

^{w,x}Within a column, means without a common superscript differed ($p < 0.05$)

^{a,b}Within a parameter, means within a row without a common superscript differed ($p < 0.05$)

Keywords: Stallion, semen, extender; cryopreservation, andrology

Sperm parameters after cushion centrifugation of stallion cooled-stored semen

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Clinical practice suggests that some stallions may benefit from semen centrifugation and re-extension after cooled storage and shipping. This study aimed to determine the effects of centrifugation and resuspension on stallion cooled semen. It was hypothesized that an egg-yolk based extender would improve sperm motilities parameters after cushion centrifugation (CC) of cooled-stored semen. Ejaculates ($n = 25$) from 5 mature stallions were extended to 25 million/ml with a skim milk-based extender (CST, Animal Reproduction Systems, Chino, CA) and stored for 24 or 48 hours in a passive cooling device (Equine-Express II; EE, Exodus Breeder, York, PA) or Equitainer (EQ; Hamilton BioVet, Ipswich, MA). After storage, semen was processed through CC ($1000 \times g$, 20 minutes), supernatant was discarded, and pellet resuspended in 2 ml of INRA 96 (IMV, Maple Grove, MN) or Botucurio (Botupharma, Phoenix, AZ). Noncentrifuged aliquots of cooled stored semen served as controls. Motility parameters were assessed with a computer-assisted sperm analyzer (Spermvision, MOFA, Verona, WI) before and after CC and in control samples that were not processed. Assessment included total motility (TM) and progressive motility (PM). Data were analyzed using a mixed model (R project) accounting for fixed (containers, time, and extenders) and random (stallion and ejaculate) effects. CC did not affect ($p > 0.05$), TM at 24 or 48 hours, whereas PM tended ($p = 0.07$) to decrease after centrifugation and 24 hours of storage (Table). Additionally, after 24 hours, none of the other sperm kinetics parameters were affected ($p > 0.05$) by the type of extender, processing with CC or the type of container. These results could either suggest that: 1) CC cannot be used to enhance sperm kinetics parameters of cooled-stored semen; 2) or it could be possible that apparent benefits of CC cannot be seen on normospermic stallions (as used herein). Thus, it is reasonable to speculate that if stallions with poor semen cooling quality were used, results may be different. In addition, CC has another benefit that was not assessed here; upon arrival, semen needs to be concentrated in a small volume for deep uterine horn insemination. Additional studies are warranted to assess effects of CC on sperm motility parameters on stallions with poor-quality semen cooled semen.

Table. Motility parameters (mean \pm SEM) after cushion centrifugation in cooled stallion semen

Time (hours)		Total motility (%)			Progressive motility (%)		
		Control	Inra96	Botucurio	Control	Inra96	Botucurio
24	EQ	64 \pm 3 ^{xa}	64 \pm 3 ^{xa}	70 \pm 3 ^{xa}	54 \pm 4 ^{xb}	52 \pm 3 ^{xb}	58 \pm 3 ^{xb}
48	EQ	54 \pm 4 ^{yb}	61 \pm 3 ^{yb}	64 \pm 2 ^{yb}	45 \pm 4 ^{yb}	50 \pm 3 ^{yb}	53 \pm 3 ^{yb}
24	EE	60 \pm 3 ^{xb}	70 \pm 2 ^{xb}	70 \pm 3 ^{xb}	51 \pm 4 ^{xb}	60 \pm 3 ^{xb}	59 \pm 2 ^{xb}
48	EE	55 \pm 4 ^{yb}	61 \pm 3 ^{yb}	64 \pm 3 ^{yb}	45 \pm 4 ^{yb}	51 \pm 4 ^{yb}	50 \pm 4 ^{yb}

EQ: Equitainer; EE: Equine-Express II

^{a,b}Within a row and parameter, means without a common superscript differed ($p < 0.05$)

^{x,y}Within a column, means without a common superscript differed ($p < 0.05$)

Keywords: Sperm motility, semen quality, horse, equine, andrology

Evaluating the impact of a systemic treatment protocol on uterine biopsy grade and uterine microbiome in mares with pretreatment Kenney grades of IIB-III

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Various treatment strategies have been utilized to correct endometritis in broodmares. Majority of described treatment protocols involve intrauterine administration of various pharmacologic compounds. One treatment protocol used by one of the co-authors, Dr. James Bailey, in embryo transfer donor mares and mares intended to carry their own pregnancies, involves a regimen of DMSO, antibiotics, and dexamethasone (all systemic treatments) administered at specific times during a course of 8 days. This treatment protocol has been anecdotally reported to produce positive results (successful embryo donors and also carrying their own pregnancies) in mares with uterine biopsy Kenney grades of IIB or III. It was hypothesized that this treatment protocol would: 1) improve Kenney biopsy grades IIB or III; and 2) alter uterine microbiome. Objectives were to evaluate the impact of this treatment protocol on each mare's: 1) endometrial biopsy grade; and 2) uterine microbiome. Mares (n = 13) with previous uterine biopsy grades of IIB-III were included. Eligibility was determined by first collecting an endometrial biopsy sample. Once mares with biopsies that were graded as IIB-III were selected and a pretreatment uterine microbiome sample was obtained using triple-guarded uterine culture swabs. Two swab samples were obtained from each mare. One swab was utilized for 16s-rDNA microbiome analysis. The other swab was submitted for aerobic and anaerobic bacterial culture. Once the pretreatment samples were obtained, the following treatment protocol began: intravenous administration of 250 ml of DMSO mixed with 750 ml of saline on days 1 and 3 (day 1 being the first day of treatment), a daily, oral antibiotic (Equisul-SDT, sulfadiazine-trimethoprim, 24 mg/kg, Aurora Pharmaceutical, Northfield, MN) on day 4 and continuing through day 8, and finally 80 mg of intramuscular dexamethasone on days 6 and 8 (day 8 was the last day of the treatment protocol). Thirty days after the completion of the treatment protocol, posttreatment uterine biopsies and microbiome swab samples were once again obtained. Results are currently pending (laboratory shutdown due to COVID-19), but will be available for presentation at the Society for Theriogenology conference in July. This study will provide objective data to characterize the impact of this treatment protocol on the endometrial grade and the microbiome of treated mares.

Keywords: DMSO, endometrial microbiome, 16s rDNA, embryo recipient, sulfadiazine-trimethoprim

Effect of platelet rich plasma lysate and fibroblast growth factor 2 on sperm motility in stallions

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Semen extenders are continually tested to improve sperm motility, longevity, and, consequently, improve fertility. Growth factors (GFs) modulate cell function, which could be advantageous to sperm by improving motility. In humans and mice, fibroblast growth factor 2 (FGF2) improved sperm motility. Platelet rich plasma (PRP), which is rich in growth factors (GFs) including FGF2, reduced postmating inflammatory response within the uterus when infused 24 hours before or after artificial insemination. Effect of PRP on sperm is not determined. Objective was to evaluate the effect of adding either pooled PRP lysate (PRPL) or recombinant equine FGF2 (reFGF2) at varying concentrations to semen extended in commercial equine semen extender (INRA 96). PRP lysate was used instead of PRP, since it is more purified and highly concentrated in GFs. Eight treatments were tested using concentrations of 1, 2.5, 5, and 10% of PRPL containing 1 IU/ml of heparin, and 0.1, 1, 10, and 100 ng/ml of reFGF2 and compared to control groups with and without 1 IU/ml of heparin. Heparin use was based on a previous titration to prevent gel formation from PRPL reaction with semen extender, precluding motility analysis. Motility parameters were evaluated with samples standardized to 50 million sperm/ml using computer assisted semen analysis (CASA) at hours 0, 0.5, 1, 1.5, 6, and 24 after treatment. For both PRPL and reFGF2 treatments, there were no differences ($p = 0.99$) in motility among groups at any time point, with ranges from 66 to 60% at hour 0 and 46 to 49% at hour 24. Interestingly, results concentrations of PRPL > 5% induced sperm agglutination via head-to-head attachment (HHA), starting at hour 1, whereas PRPL at concentrations below 2.5% did not induce HHA nor affect sperm motility. In addition, HHA was objectively detected by decreases in total number of cells counted per field (total cells) and estimated concentration measured by CASA, assuming that only free sperm were counted, whereas sperm entrapped by HHA not counted. Decreases in total sperm and concentration were different ($p < 0.0001$) for 5 and 10% PRP groups, being more pronounced for 10% PRPL group, suggesting a dose-dependent characteristic of HHA induced by PRPL. One important finding was that PRPL did not kill sperm, besides inducing HHA. Although no motility improvement was observed, present results suggests that direct addition of PRPL in semen extender at doses below 5% could be implemented to reduce postmating uterine inflammatory response, without substantially affecting sperm motility. However, further research on uterine inflammatory response is needed to test this hypothesis.

Keywords: Growth factors, sperm motility, endometritis, head-to-head attachment

Prevalence of and potential impact on fertility of pars pituitary intermedia dysfunction in a Thoroughbred broodmare population in England

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Prevalence of pars pituitary intermedia dysfunction (PPID) has been reported in general equine population as 21.2% in horses and ponies aged ≥ 15 years.¹ Aim was to establish the prevalence of PPID in a Thoroughbred broodmare population using seasonally adjusted cutoff values for basal plasma adrenocorticotrophic hormone (ACTH) concentrations and analyze the potential impact on fertility. Venous blood samples were collected from 79 Thoroughbred broodmares ≥ 15 years old (average 18 years) on first January 2019. Samples were analyzed for plasma ACTH concentrations using Immulite 1000 assay. Mares with plasma ACTH concentrations above the seasonally adjusted reference range for nonautumn months (> 29.7 pg/ml) were allocated into a followup group and underwent repeat sampling in 2019 autumn. Prevalence of PPID in January 2019 was 16.4% (13/79), with mean plasma ACTH concentrations of 38.0 pg/ml (30.2 - 61.4) in affected mares. On repeat sampling, 23% (3/13) mares remained positive (> 47 pg/ml; autumn reference range), whereas 46% (6/13) had a reduction in basal plasma ACTH concentration. Furthermore, 7% (1/13) had small increases in plasma ACTH that was still within normal limits for autumn, whereas 23% (3/13) were lost to followup. Mean pregnancy rate per cycle for 13 mares in the followup group was 52.4%. Five mares were not bred in the 2019 breeding season. Two pregnancies were lost between 15 and 28 days. One mare was lost to followup. Initial prevalence of PPID within this population was comparable to previous studies; however, followup data in autumn had a decrease. Prevalence of PPID in this study population was lower than general equine population, with elevated ACTH concentrations having no impact on fertility. Affected mares had consistent estrous cycles and were able to conceive and maintain pregnancy to 28 days at a pregnancy rate comparable to previous reports for aged mares. Furthermore, these results also highlighted the requirement for repeat testing and need for further investigation into use of single basal plasma ACTH concentrations as a conclusive test for PPID.

Keywords: Equine, PPID, ACTH, fertility, prevalence

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**Kisspeptin and RFamide-related peptide 3 neurons in bovine hypothalamus:
estrogen receptor α expression and inputs to gonadotrophin releasing hormone neurons**

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Fertility in dairy cattle is considered low, most likely due to selection for high milk production resulting in extreme metabolic demands and a period of negative energy status. In most mammals, there appears to be an inverse relationship between metabolic status and reproduction. To address and manage this relationship in dairy cows, a better understanding of brain control of reproduction is required. Final output signal of the brain is gonadotropin releasing hormone (GnRH), but it is evident that integration of internal and external signals, such as feed intake and metabolic status, occurs upstream of the GnRH neurons. The RF-amide neuropeptides, kisspeptin (Kp), and RFamide-related peptide 3 (RFRP-3) may be involved in this integration and relaying this to the GnRH neurons. In cattle, GnRH secretion is stimulated by Kp and inhibited by RFRP-3. We used immunohistochemistry (IHC) to map the distributions of Kp and RFRP-3 neurons in the hypothalamus of dairy cattle, determined their co-expression with estrogen receptor alpha (ER α), and whether they made contact with GnRH neurons. The heads of male and female calves (n = 3 each), steers, heifers, and lactating cows (n = 2/group) were perfusion-fixed (4% paraformaldehyde) and the brains dissected out and frozen. Dual-label IHC for Kp or RFRP-3 with ER α or GnRH was performed on 40 micron thick cryostat sections, using fluorescently labelled secondary antibodies. GnRH neurons were identified in the preoptic area and anterior hypothalamic area. Highest numbers of neurons expressing ER α were in the arcuate nucleus, ventromedial nucleus, preoptic area, and anterior hypothalamic area. Kp neurons were located primarily in arcuate nucleus, with some cells in the preoptic area (although few were observed in calves). Most Kp neurons in adult animals of both sexes, but few in calves, expressed ER α . Very few GnRH neurons, however, received close appositions from Kp neuron fibers. RFRP-3 neurons were localised in the dorsomedial hypothalamus and paraventricular nucleus, with fewer cells observed in cows than heifers and steers, whereas ER α was not expressed in these regions. Fewer than 20% of GnRH neurons received close contact from RFRP-3 neurons. In conclusion, we inferred that feedback actions of estrogen on GnRH secretion may, in part, be relayed via Kp neurons but unlike most other mammalian species studied, this is unlikely to be a direct action. RFRP-3 neurons likely have a small role in the regulation of GnRH neurons in cattle. Further studies are required to clarify the physiological roles of Kp and RFRP-3 in regulation of GnRH secretion in dairy cattle.

Keywords: Kisspeptin, dairy cattle, GnRH, hypothalamus, neuroendocrine signalling

Oviductal insemination by hysteroscopic hydrotubation in mares: a preliminary investigation

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Small numbers of sperm were recovered from the oviduct after natural mating or artificial insemination in mares. One advantage of oviductal insemination is that only small number of sperm may be required to achieve pregnancy in a mare compared to numbers required with standard intrauterine insemination. Objective was to investigate the potential for oviductal insemination, using hysteroscopic hydrotubation¹ with small number of sperm. We hypothesized that pregnancy can be established with a small number of sperm, obtained from frozen semen and infused into the oviduct directly through the uterotubal junction by hysteroscopic hydrotubation. Ten mares were used; 5 mares each were assigned into 2 groups and inseminated when preovulatory follicle size reached 35 - 40 mm. Ovulation was confirmed 48 hours after insemination. Progressively motile sperm were selected using the swim-up technique with frozen semen of known fertile stallions (A and B). One straw of 0.5 ml frozen semen was thawed and mixed with 0.5 ml Quinn's[®] Sperm Washing Medium and 1 ml of the same medium was floated onto the mixed solution and incubated for 10 minutes at 37°C. Number of sperm that swam up into the supernatant was counted with a Thoma cytometer. Progressively motile sperm concentrations were 1.3 and 1.2 x 10⁵/ml in the supernatant of Stallions A and B, respectively. In Group 1 (5 mares) frozen semen of Stallion A was used. From 0.1 to 1.0 ml of the supernatant including sperm was mixed with 1.0 or 2.0 ml of the same medium to adjust the number of sperm (1.3, 2.0, 2.0, 4.1, 4.1, and 9.8 x 10⁴) and total volume of medium (1.1, 1.7, 1.7, 1.9, 1.9, and 1.5 ml). Medium, including sperm, was infused into oviduct through uterotubal junction ipsilateral to ovulation site using hysteroscopic hydrotubation. One of 2 mares was pregnant in cases where 2.0 and 4.1 x 10⁴ sperm were used, 1 mare was pregnant with 9.8 x 10⁴ sperm. In Group 2 (5 mares), 1.9 x 10⁴ sperm from Stallion B in 1.7 ml medium was infused into oviducts in 6 cycles of 5 mares by the same procedure used in Group 1. Three of 5 mares (3/6 cycles) in Group 2 were pregnant. In conclusion, oviductal insemination of small number of sperm to achieve pregnancy in mares using hysteroscopic hydrotubation may be a clinically applicable technique.

Keywords: Oviductal insemination, hysteroscopic hydrotubation, mare

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Effect of mid-diestrus oxytocin treatment on early pregnancy in mare

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Progesterone supplementation in early pregnancy is commonplace in equine reproduction. Recently, there has been some controversy questioning the beneficial effects of routine progesterone administration and rising concerns as an occupational hazard for humans. Although premature luteal regression and resulting low plasma progesterone concentrations is rare in the mare, select cases benefit from luteal support. Luteal function can be extended following oxytocin administration during mid-diestrus. Aim of this field study was to investigate the effects of oxytocin administration on early pregnancy in the mare. Thoroughbred mares ($n = 39$) were enlisted and bred over 55 estrous cycles. Prior to natural breeding, uterine cultures were obtained and mares were assigned to 1 of 2 groups: Oxytocin treatment (OT); oxytocin 60 units, IM, once daily, days 7 - 14 postovulation; or Control (CT), given no treatment. Mare reproductive cycles were monitored by transrectal palpation and ultrasonography. Ovulation was documented and defined as day 0. Pregnancy diagnosis was performed by transrectal ultrasonography 14 days postovulation. If pregnancy was confirmed, a blood sample was collected and assayed for plasma progesterone concentrations. Mares that did not conceive were rebred and treatment was determined by the stage in the study; therefore, some mares underwent the same treatment more than once. Subsequent pregnancy examinations were performed on days 28 and 45 of pregnancy. First and percycle pregnancy rates for OT and CT were 90.9 and 91.3% and 68.2 and 55.1%, respectively. Median progesterone concentrations for OT were 6.9 and 7.0 ng/ml for CT. There were no differences between OT and CT groups for first ($p = 0.165$) or percycle ($p = 0.289$) pregnancy rates, days 28 and 45 pregnancy rates ($p = 1.0$) or progesterone concentrations ($p = 1.0$). Oxytocin administration during mid-diestrus in early pregnancy did not induce a negative effect. Further investigation in this area may reveal potential beneficial effects of oxytocin administration in early pregnancy.

Keywords: Mare, oxytocin, corpus luteum, progesterone, breeding management

Combination of estradiol cypionate and altrenogest to control ovulation timing in mares

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Controlling ovulation timing in mares is used primarily for convenience when frozen or transported semen is used, or to provide recipients for an embryo transfer program. Luteal or progestational phase termination with prostaglandin F_{2α} or its analogs, results in too variable a time to ovulation to be of practical use (3 - 15 days). Progestogens alone, although capable of inhibiting ovulation, have no control on follicular development. Time from withdrawal of progestogen to ovulation is too variable to be useful in timed insemination with frozen semen or embryo reception. A combination of progesterone and estrogen (P & E) to inhibit both follicle stimulating hormone (FSH) and luteinizing hormone (LH) synthesis and release results in more precise control of follicular development and ovulation. This protocol requires daily intramuscular (IM) injections and use of compounded products. Estradiol-17 β is reported to be the best product, because estradiol cypionate (ECP) and estradiol benzoate are too slowly metabolized and cause delayed or erratic return to estrus. Anecdotal reports dispute this claim. Goal of this study was to determine the usefulness of a combination of commercially available products and minimize the number of IM injections to control ovulation in the mare. Procedures were approved by Oregon State University Animal Care and Use Committee. Ten mares of variable age (17 - 23 years, mean 19.7 years) and size (515 - 626 kg, mean 570 kg) were given altrenogest liquid (Regumate[®], Merck Animal Health, Madison, NJ) 0.044 mg/kg orally, once daily, for 10 days and estradiol cypionate (Depo Estradiol[®], Pfizer, New York, NY) 0.011 mg/kg IM on day 1 and day 5. Study was performed during normal breeding season for the latitude. None of the mares were examined prior to treatment to determine stage of estrous cycle. Mares were evaluated by transrectal ultrasonography of reproductive tract, had serum progesterone concentrations measured, and received cloprostenol (Estrumate[®], Merck Animal Health, Madison, NJ) 0.5 μ g/kg IM on day 10. Reproductive examinations were performed daily or on alternate days, from days 15 or 16, to detection of ovulation. Nine out of 10 mares had progesterone concentrations < 1 ng/ml at the end of 10 day study periods. Largest follicle at cloprostenol treatment was 38 mm (< 20 - 38 mm) and second largest was 27 mm. Eight mares ovulated 7.25 - 7.625 days after discontinuation of altrenogest. Even a mare with 38 mm follicle did not ovulate until day 17, despite having < 0.2 ng/ml progesterone at cloprostenol treatment. Two mares did not ovulate within 14 days of discontinuation of treatment. It is concluded that ovulation timing can be controlled in mares using commercial compounds readily available to the practicing veterinarian, although fertility of those ovulations remains to be determined.

Keywords: Mare, ovulation, ECP, altrenogest

Prevalence of malignancy in canine mammary masses in a population of shelter dogs

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A retrospective study was carried out on 11 bitches with mammary masses that were surrendered to a municipal shelter in Florida from 2017 to 2019. Ten bitches had reached sexual maturity, and 1 did not have an indicated age. Objective was to determine the prevalence of malignancy of mammary masses in this population. Mammary masses were removed at ovariohysterectomy and submitted to a commercial pathology service for analysis. Ten bitches had solitary mammary masses and 1 had 2 mammary masses. A total of 12 mammary masses were submitted; 11 (92%) had no evidence of malignancy and 1 mass (8%) had low-grade neoplasia. Most common masses identified were adenomas ($n = 7$). Other masses identified included a parasitic granuloma, follicular cyst, lipoma, and mammary ductal ectasia. One neoplastic mass (grade 1 mammary carcinoma identified) was excised from a 7 year old Chihuahua mix with multiple mammary masses. Three (25%) masses in this patient were not mammary in origin. Chihuahuas and Chihuahua mixes ($n = 6$) were overrepresented in this study. In this small population, mammary masses were predominantly benign. Further studies are needed with expanded sample size and involving a reproductive pathologist.

Keywords: Mammary, neoplasia, canine, benign, malignant

**Intravaginal progesterone releasing device to hasten first ovulation in mares:
side effects and pregnancy rates in a commercial Thoroughbred farm**

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Many protocols have been evaluated to hasten first ovulation of breeding season in mares, including use of progesterone releasing devices. However, use of these devices in clinical practice is not widespread. In the Thoroughbred industry, there is immense pressure to have mares cycling in the nonphysiologic breeding season. Thoroughbred mares (n = 20) over 2 consecutive breeding seasons in a commercial studfarm underwent treatment with a PRID™ Delta (1.55 g progesterone) inserted intravaginally for 10 days. Mares (5 - 23 years) were not under artificial lights. Mares at time of insertion were deemed to be in early transition via transrectal ultrasonography, having at least 1 follicle of 2.0 cm, no uterine edema, and no previous ovulation noted for that breeding season. On removal of PRID™ Delta, mares had a preovulatory follicle of at least 3.5 cm. On day 11 (1 day after removal), mares had a uterine edema score of at least 1 (on a scale 0 - 3) and were given deslorelin acetate to induce ovulation. All mares (100%) ovulated within 3 days after removal of PRID™ Delta (day 13). Eight mares (40%) cultured positive for endometrial *Streptococcus equi* subspecies *zooepidemicus*. Remaining 12 mares (60%) were inseminated via live cover, resulting in 10 established pregnancies (50%) diagnosed on day 14 by transrectal ultrasonography. No vaginal discharge was observed in any of the mares, although all mares were noted to have some increase in vascularization of the vaginal wall with some hyperemia (n = 20). No other complications were noted in the reproductive tract of any of the mares. Discomfort upon removal of the device, including straining, mild colic-like signs, and weight shifting, were noted in some mares (6/20) all of which were transient in nature, and required no medical interventions. It is concluded that PRID™ Delta use to hasten first ovulation in mare is effective with minimal side effects and results in a fertile ovulation. Use of intravaginal progesterone releasing devices along with other methods to manipulate mare's physiologic breeding season, such as artificial lighting regimes, can hasten time to first ovulation and reduce associated costs.

Keywords: Intravaginal progesterone, breeding management, mare, first ovulation

Individual variation of frozen-thawed sperm from stallions to survive dilution and cooling after thawing

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Within the equine industry, there has been interest in the ability to thaw frozen semen in a semen production laboratory, dilute further in extender and ship it as a cooled dose to the farm for insemination. Two previous studies concluded that acceptable retention of motility and fertility can be achieved using this technique, but was only done on a small set of samples. Goal of this experiment was to measure semen quality after thawing and cooling over 48 hours on a large number of stallions and illustrate the variability in the success among individual stallions. We evaluated 22 doses of frozen semen of variable post-thaw quality, all of which previously resulted in pregnancies. Motility analyses were performed using a Hamilton Thorne Ceros II computer assisted sperm analysis system to evaluate at least 400 motile sperm from each sample at each time point. Samples were analyzed for 40 video frames at 60 frames/second and progressive motility (PM) was defined as motile sperm moving with an average path velocity $\geq 50 \mu\text{m}/\text{second}$ and a straightness value of $\geq 75\%$. A full dose of eight (0.5 ml) straws per sample were thawed at 37°C for 30 seconds, then combined in a prewarmed 5 ml tube. Concentration was determined with a NucleoCounter SP-100 and an aliquot of the sample was added to pre-warmed cooling extender to a concentration of approximately 10 million/ml. After incubation for 30 minutes at 37°C , initial motility was determined. A second aliquot of thawed sample was incubated in modified Whitens medium with SYBR-14 and propidium iodide at a concentration of 5 million/ml. Membrane integrity analysis was completed on a Guava EasyCyte HT flow cytometer. Frozen-thawed samples were diluted to 30 million/ml in extender previously determined to be optimum for cooling semen of that individual stallion (INRA96[®] or a standard skim milk-glucose extender). Motility and membrane integrity analyses were performed initially (time 0), 6, 10, 24, 32 and 48 hours postthaw. Percent change in motility and membrane integrity was calculated from time 0 (initial) to 6, 24, and 48 hours after cooling. Average percent decrease in PM across all 22 stallions was 23, 41, and 67% at 6, 24, and 48 hours. Average percent decrease in sperm with intact membranes was 16, 16, and 23% at 6, 24, and 48 hours. Individual variation in the success of this technique was substantial. Decrease in semen quality after thawing and cooling for 24 hours, ranged among stallions from 2.5 to 77.6% for PM and 0 to 37% for membrane integrity. Decrease in PM for top 5 stallions at 6, 24, and 48 hours were 5, 13, and 33% respectively whereas for bottom 5 stallions there was a 47%, 71%, and 98% change in PM, respectively. It is concluded that although this technique is a viable option for some stallions, it is not appropriate for all stallions and therefore must be tested on every stallion.

Keywords: Equine sperm, frozen-thawed, cooled, membrane integrity

**Comparison of pregnancy rates using new versus once used CIDR
in 7 day estrus synchronization protocol during breeding season in ewes**

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Small ruminant CIDRs are labelled for use in estrus synchronization protocols for up to 14 days. Protocols of shorter duration (5 - 9 days) include prostaglandin F_{2α} (PGF_{2α}) treatment at CIDR removal to induce luteolysis. Short duration protocols have led to anecdotal belief that a CIDR may be used a second time. Addition of gonadotropins to CIDR based protocols are known to enhance follicular growth and facilitate multiple ovulations. Our objective was to compare pregnancy status following natural breeding at a synchronized estrus using a 7 day exposure to either a new CIDR or once used CIDR, with PG600 treatment on day 6. Canadian Arcott ewes (mixture of lambs and mature) housed at 2 separate locations (Flocks A and B) were used. In Flock A, ewes (n = 61) were synchronized during 2018 - 2019 (n = 31) and 2019 - 2020 (n = 30) breeding seasons, whereas ewes (n = 71) in Flock B were synchronized during 2018 - 2019 breeding season. On day 0, ewes were randomly allocated to have either a new CIDR (nCIDR) or used CIDR (uCIDR) placed intravaginally. On day 6, all ewes were treated with PG600 (400 IU eCG + 200 IU of hCG/5 ml dose, IM). On day 7, CIDRs were removed and all ewes received a single dose of PGF_{2α} (cloprostenol, 125 mg, 0.5 ml, IM). On day 8, ewes were exposed to rams for 4 days. Rams were identified as satisfactory breeders using breeding soundness evaluation and previous breeding history. Ram to ewe ratio was maintained at 1:5 (Flock A) and 1:5 to 1:6 (Flocks B). Rams were provided 4 - 5 days of sexual rest before being used. Pregnancy diagnosis was performed between days 45 - 60 postbreeding via transabdominal ultrasonography. Fischer's Exact test was used to compare pregnancy rates between treatment groups. All ewes in Flock A were marked (ram marking harness) within 24 hours after ram introduction. Overall, pregnancy rates (nCIDR and uCIDR combined) were different (p < 0.01) between flocks (85%, 52/61 versus 58%, 41/71; Flocks A versus B, respectively); therefore, data were analyzed separately by flock. Within flock, pregnancy rates were not different between treatment groups (Flock A: nCIDR versus uCIDR, 25/30 versus 27/31, p = 0.75; Flock B: nCIDR 21/34 versus 20/37, p = 0.63). Lower pregnancy rates in Flock B were believed to be due to nutritional status (as a general observation, body condition scores of ewes in Flock B were lower than those in Flock A). In conclusion, a short duration (≤ 7 days) once used CIDR, can be successfully reused to obtain pregnancy rates comparable to new CIDR.

Keywords: Estrus synchronization, PG600, CIDR, ewes, fertility

Retrospective review of uterine prolapse in mares

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Uterine prolapse (eversion) is an infrequent but life-threatening emergency in mares. Since uterine prolapse is so uncommon, there is very little information published in scientific journals. Aim of this report is to describe uterine prolapse predispositions and case outcomes by reviewing clinical records from the UC Davis Veterinary Medicine Teaching Hospital (VMTH). Mare history and outcome data were analyzed for correlation to mare survival with Mann-Whitney U calculation and Fishers Exact Test. Significance level of $p < 0.05$ was set for all analyses. During 30 year period (1989 - 2019), 24 mares were presented with uterine prolapse. Age of mares ranged from 4 - 23 years (Mean \pm SD; 11.1 ± 4.6) and parity ranged from 1st to 13th foaling (Mean \pm SD; 3 ± 2.7). There was a high representation of maiden mares (7 out of 24). Investigation of interrelation between breed and uterine prolapse revealed that Arabians were overrepresented among affected mares compared to general breed distribution at the VMTH in the same time period. Uterine prolapse was associated with parturition and occurred within 1 hour after foaling in 82% of the cases. Fetal membranes were attached at the time of prolapse in 63% of the cases. Contrary to common belief that dystocia is the greatest predisposing factor to prolapse, only 33.3% of prolapses occurred after dystocia and 16.6% prolapses followed an abortion. Survival of the mares at discharge was 75%. Regarding fatalities, 5 mares suffered acute hemorrhage associated with prolapse and 1 died from secondary peritonitis. Fetal gender was overrepresented by males, 12 foals were colts and 5 were fillies (7 unknown). Fifteen foals survived. We were able to obtain follow-up history for 14 mares; 5 were rebred, and 4 foaled successfully. There were no correlations between mare survival and parity, age, sex of the foal, retained fetal membranes, or occurrence of sepsis. Based on the findings, we concluded that uterine prolapse occurred more after normal parturition (eutocia) than following abortions (16.6%) and dystocia (33.3%). Uterine prolapse may also have some breed predisposition to Arabians. Colts are overrepresented among affected mares. No characteristics were correlated with survival of mare, but severe hemorrhage was correlated with nonsurvival.

Keywords: Mare, uterine prolapse, normal parturition, abortion, dystocia

Alfaxalone crossreactivity affecting progesterone concentrations in cats

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Alfaxalone is a commonly used anesthetic agent in small animals. In cats, alfaxalone is used as an intramuscular agent to achieve clinically useful sedation or anesthesia, negating need for intravenous treatment, particularly in difficult patients. Molecular structure of alfaxalone is similar to progesterone (P_4) hormone allowing for activation of $GABA_A$ complex through a progesterone receptor. On clinical observation, intact female cats demonstrating signs of estrus, following alfaxalone treatment had serum P_4 concentrations suggestive of luteal activity. Concern that alfaxalone may be interfering with the assay, we hypothesized that alfaxalone would crossreact with progesterone assay. Eight domestic shorthair neutered male cats were administered 3 mg/kg of alfaxalone IM. Blood samples were collected at set time points (baseline, 30 and 60 minutes, and 3, 6 and 10 hours). Serum concentrations of P_4 immunoreactivity (IR) were determined using an automated immunoassay system (Immulite 1000). Data were analyzed using repeated measures ANOVA and a Tukey-Cramer multiple comparisons test, with $p < 0.05$ used for significance. Serum P_4 IR was elevated ($p < 0.05$) at 30 minutes, 1 hour, and 3 hours compared to baseline and returned to baseline at 6 hours. We concluded that intramuscular alfaxalone treatment in cats may interfere with immunoassay measurement of serum P_4 for up to 6 hours. Caution should be exercised when interpreting serum P_4 IR results in cats treated with alfaxalone.

Keywords: Alfaxalone, progesterone, immunoreactivity, cat

Influence of extender, temperature, and equilibration time on postthaw sperm motility in ram semen

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Objective was to investigate effects of extender, loading temperature, and equilibration time on postthaw sperm motility. Ejaculates were collected with an artificial vagina from 4 rams from May - July. Each ejaculate was split into 3 aliquots and extended in a 1 step protocol to a concentration of 200×10^6 sperm/ml with a liposome-based extender (OPTIXcell™), an egg yolk-based extender (Trilady1®), and a soy lecithin-based extender (AndroMed®). Half of each extended aliquot was loaded into straws at ambient temperature (23°C) and left to equilibrate for either 2, 4, or 12 hours at 4°C before cryopreservation in liquid nitrogen. Remaining half was loaded at 4°C after equilibration before freezing. Concentration and motility were measured with iSperm® Semen Analyzer. Linear mixed models were used for statistical analysis. Results are expressed as least square means \pm SEM. Postthaw sperm motility differed ($p < 0.0001$) among OPTIXcell™ ($37.1 \pm 1.5\%$), AndroMed® ($26.3 \pm 1.8\%$), and Trilady1® ($30 \pm 0.8\%$) extenders. Ambient loading temperature ($32.6 \pm 1.5\%$) also had appreciable positive effects ($p < 0.0147$) on postthaw motility compared to 4°C ($29 \pm 1.1\%$). Samples with an equilibration time of 2 hours had lowest ($p < 0.0001$) postthaw sperm motility ($23 \pm 1.2\%$) compared to 4 hours ($33.8 \pm 2.3\%$) and 12 hours ($35.6 \pm 1.5\%$) equilibration. However, sperm postthaw sperm motility between 4 hours and 12 hours equilibration was not different ($p < 0.4270$). In conclusion, it is optimal to extend ram semen with OPTIXcell™ to avoid risk of microbial contamination that comes with animal-based extenders, load at ambient temperature which is less labor intensive and may limit possibility for temperature fluctuations, and to equilibrate at 4°C for 4 hours which could lengthen the window of AI postthaw. This protocol is ideal for efficiency and attractive to those without experience or cuttingedge equipment to freeze semen.

Keywords: Extender, cryopreservation, ram semen, motility, iSperm, temperature

Sonographic appearance of late pregnancy sheep fetal intestine and kidney

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Objective was to identify changes in sonographic characteristics of late pregnancy ovine fetus associated with fetal maturity. Ewes were synchronized, ram bred, and confirmed pregnant (n = 18) for potential cesarian section (c-section) at day 145 of pregnancy (d 0). Transabdominal ultrasonography was performed at days (d) 22, 15, 10, 7, 4, 1, and 0 prepartum to assess fetal heart rate (HR), abdominal diameter, renal volume, corticomedullary distinction (CMD), and gastrointestinal peristalsis (GIP). At day 1, 12 ewes were induced with 20 mg dexamethasone IM and underwent elective c-section on day 0. At birth, lambs were assigned a vigor score (1 - 10) based on HR, respiratory rate, muscle tone, irritability reflex, and mucous membrane color. A total of 19 live and 3 dead lambs were delivered with an average vigor score of 7 ± 0.6 . Statistical analyses were performed using R. Mean days prepartum were assessed using ANOVA. Pearson's correlation analysis was used to assess associations. Both CMD and GIP were strongly correlated with days prepartum ($r = 0.7$, $p < 0.001$), whereas low correlation was observed with renal volume ($r = 0.27$, $p = 0.006$) and abdominal circumference ($r = 0.25$, $p = 0.009$). Days prepartum differed for CMD categories (absent: 22 ± 0 , indistinct: 9.1 ± 0.6 , distinct: 0.5 ± 0.1 ; $p < 0.001$). Days prepartum also differed for GIP categories (no peristalsis: 12 ± 0.7 , occasional peristalsis: 2.3 ± 0.4 , frequent peristalsis: 0.6 ± 0.1 ; $p < 0.001$). Days prepartum did not differ when fetal HR measured < 130 bpm (7.1 ± 0.7) or $130 - 149$ bpm (8.8 ± 1.1 ; $p = 0.43$), but differed when fetal HR measured > 150 bpm (15 ± 1.5 ; $p < 0.001$). Fetal HR prior to induction (day 1) was moderately correlated with vigor scores at birth ($r = 0.55$, $p = 0.07$). In summary, qualitative sonographic characteristics, such as onset of fetal GIP and CMD were superior to quantitative measurements in determining fetal maturity. Additionally, ultrasonographic assessment of fetal HR before induction may help to predict neonate survivability.

Keywords: Ewe, pregnancy, GI peristalsis, renal development, ultrasonography

Efficacy of deslorelin acetate in advancing ovulation in timed artificial insemination in goats

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Objective was to determine the efficacy of deslorelin acetate in advancing ovulation for use in timed artificial insemination (TAI) protocols in goats. Does received an intravaginal CIDR containing 0.3 g progesterone and 10 mg dinoprost tromethamine IM at day -5. After CIDR removal (day 0), does received 0.2 mg deslorelin acetate (n = 9) or saline (control, n = 10) IM. Transrectal ultrasonography was performed twice daily to measure follicular growth and determine time to ovulation. Subsequently, does were synchronized similarly and left untreated (control, n = 42), administered 0.2 mg deslorelin IM (n = 42), or administered 5 ml P.G. 600[®] IM (n = 42). TAI was performed 48 - 56 hours after CIDR removal using fresh semen from 1 of 3 bucks, randomly distributed among treatments. Does were then housed with assigned bucks for natural breeding. Transabdominal ultrasonography was performed at 50 and 90 days after TAI to assess pregnancy status. Statistical analyses were performed using R. Time to ovulation was analyzed using a Kruskal-Wallis test and number of ovulations was analyzed by Student's t-test. Pregnancy rates were analyzed by ANOVA using treatment and buck as variables. Deslorelin-treated does had increased (p < 0.01) number of ovulations (2.9 ± 0.4) over control does (1.5 ± 0.2). Hours to ovulation tended to be less in deslorelin-treated does (53 ± 11) than in control does (61 ± 11; p = 0.06). Pregnancy per AI was affected by both treatment (p < 0.01) and buck (p = 0.04). Control does bred by Buck 1 had higher (p = 0.02) percent pregnancy rates (50) than those treated with deslorelin (7) or P.G. 600[®] (14). Deslorelin-treated does also had decreased (p < 0.01) in overall pregnancy rates across all bucks. It is concluded that deslorelin may have superovulatory effects impacting does' cyclicity and ability to achieve pregnancy. Further research is needed to investigate use of lower doses of deslorelin in goat estrus synchronization programs.

Keywords: Artificial insemination, caprine, deslorelin, estrus synchronization, pregnancy

Monozygotic twins in a Thoroughbred mare bred by natural cover

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Twin pregnancies in mares are undesirable, with a majority resulting in abortion or neonatal death.¹ Most twin pregnancies are dizygotic and originate from 2 oocytes rather than division of an embryo after fertilization (monozygotic). The exact etiology of monozygotic twin occurrence is not well understood in humans^{2,3} or horses.⁴ It is known, however, that although the natural occurrence of monozygotic twins is rare in both horses and humans (only 0.4% of in utero conception pregnancies in humans), the rate more than doubles when using assisted reproductive technologies.^{2,5,6} A 13 year old Thoroughbred mare was evaluated by transrectal ultrasonography while in estrus and was bred naturally (natural cover) the same day a 40 mm follicle was identified. A single ovulation was detected by ultrasonography on the following day. At 14, 15, 16, 20, 26, and 30 days postovulation, a single embryo presence was observed. On day 33, 2 heartbeats (apparently from 2 embryos) were noticed and the vesicle measured 36 mm. Presence of 2 embryos was confirmed on day 34. Due to the inherent risks of twinning, her uterus was lavaged to remove and recover the embryos for analysis. Two distinct embryos were present within a single chorionic membrane. Tissue samples from both embryos were analyzed for genetic markers, specifically 13 microsatellites, and both embryos had identical genotypes. This case of monozygotic twins was unusual in that it was not associated with assisted reproductive technologies, but with natural cover.^{1,7} Unlike the diagnosis of dizygotic twins, which can be determined around 14 days postovulation, monozygotic twins are often not diagnosed until around 30 days postovulation.⁸ It is therefore important that mares diagnosed with single vesicles early in pregnancy, be re-examined ~ 30 days postovulation to ruleout the presence of monozygotic twins.^{8,9} Even though monozygotic twins have been successfully reduced later in pregnancy,¹⁰ most likely both embryos will be lost.

Keywords: Mare, Monozygotic twins, natural cover

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Pregnancy toxemia in the bitch

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Canine pregnancy toxemia can be a life-threatening condition for the dam and fetuses. The condition is characterized by hypoglycemia, ketonemia, ketonuria, and hepatic lipidosis during late pregnancy and clinical signs include weakness, collapse, seizures, and coma. Pregnant bitches bearing a large litter are more predisposed, especially if they develop anorexia during the last 2 weeks of pregnancy. The diagnosis is made by association of clinical signs, hypoglycemia and, importantly, high concentrations of ketone bodies in the blood and urine.¹⁻³ Measurement of ketone bodies (β -hydroxybutyrate) is commonly performed in food animal medicine; however, not often in small animal practice, which may impair the diagnosis of pregnancy toxemia. If diagnosed early, the condition can be resolved with enteral and/or parenteral glucose supplementation. However, in severe cases, pregnancy termination may be required. A 2 year old female Labrador retriever presented with 1-week history of vomiting and severe lethargy. The bitch had been accidentally bred 65 days prior to presentation. Tachypnea, distended abdomen, and engorged mammary glands were noted initially on physical examination. Hypoglycemia (38 mg/dl) and hypocalcemia (9.1 mg/dl) were observed. Serum ketone measured 1.0 mmol/l (normal range, 0.0 - 0.1 mmol/l). Plasma progesterone concentration was 1.07 ng/ml. Twelve fetal skeletons were seen radiographically. On ultrasonography, fetal viability and maturation were confirmed by fetal heart rates, presence of well-defined fetal renal corticomedullary distinction and intestinal peristalsis. Based on those findings, cesarean section was elected. Surgery was performed without complication and 12 healthy puppies were successfully delivered. Dam received Vetivex[®] (lactated Ringer's with 2.5% dextrose) and 22 mg/kg calcium gluconate. This case highlighted the importance of maintaining proper nutrition and energy balance in late-term pregnancy and measuring ketone bodies in an anorexic pregnant bitch.

Keywords: Canine, pregnancy, hypoglycemia, ketonemia

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Dystocia due to congenital hydrocephalus in a miniature Dachshund

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Congenital hydrocephalus (CH) is an active distension of the ventricular system that develops because of either an interruption of cerebrospinal fluid (CSF) flow or absorption. Chihuahua, Pomeranian, Pug, Pekingese, English Bulldog, Lhasa Apso, Yorkshire, Boston, and Maltese Terrier breeds are predisposed to CH.¹ Although Dachshunds have a relatively low frequency of congenital defects compared to other breeds,² CH has been previously reported.³ However, there have been no published reports of CH resulting in dystocia in this breed. An 8 year old multiparous miniature Dachshund presented with a dystocia persisting for > 8 hours. The bitch was clinically stable on physical examination and a digital vaginal examination revealed no fetal parts. Transabdominal ultrasonography of uterus revealed 4 fetuses with heart rates between 150 - 250 bpm. A catheter was placed in the right cephalic vein and the bitch was anesthetized with propofol. Anesthesia was maintained with sevoflurane. A routine hysterotomy was performed and all 4 fetuses were delivered alive, including a fetus with a large, dome-shaped head that was lodged in the cranial vagina. No other congenital defects were present during gross postnatal examination of pups. Puppies were followed to 9 weeks of age. The pup with the CH developed normally and displayed no neurologic signs other than a consistent lack of menace reflex. Transfontanelle ultrasonography at 4 weeks of age revealed the lateral ventricles were greatly distended with anechoic fluid and lined by a thin wall of cortical tissue. At 9 weeks of age, the pup (2 kg) began acutely and inconsolably crying and began circling with head pressing. Euthanasia was performed and necropsy findings revealed 5 open fontanelles, occipital dysplasia, and symmetrical ventriculomegaly with periventricular cortical atrophy. Prior to ossification of the cranial sutures, CH may cause abnormalities of skull development such as a thinning of the bone structure, a dome-shaped appearance to the head, and/or persistent fontanelles.¹ In the current case, each ventricle contained approximately 10 ml of cerebrospinal fluid. Concurrent congenital abnormalities of the brain (e.g. intracranial arachnoid cyst, Dandy-Walker syndrome, Chiari-like malformation) without CE were previously reported in dogs.⁴ No cerebellar or other abnormalities were present grossly and there was no histological evidence of neuronal degeneration or inflammation. The most common reported malformation in CH is a stenotic mesencephalic aqueduct,⁴ which was the presumed cause in the current case, since no other lesions were present. In the current case, the formation of CSF must have equilibrated with absorption, resulting in a compensated hydrocephalic state, until an acute blockage of the ventricular system occurred, resulting in rapid decompensation.

Keywords: Canine, cranial deformity, dog, fetus, occipital dysplasia

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Fetal mummification and abortion secondary to umbilical cord torsion

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A 14 year old, Quarter Horse, mare presented, having aborted the previous day. Mare was bred by natural service and confirmed pregnant at ~ 30 days of pregnancy. Fetal crown rump length measured 24.8 cm and fetus weighed 318 grams. Estimated fetal age was 4 - 5 months. Fetus was partially mummified and visceral organs were autolyzed and appeared tan. There were > 20 counter-clockwise twists in the umbilical cord, extending from umbilicus to allantois. Umbilical cord torsion is considered a major cause for noninfectious abortion in mares. Reported frequency of abortion secondary to umbilical cord torsion varies, likely reflecting regional differences, as well as management differences leading to increased reporting. According to a retrospective study in UK, umbilical cord torsion accounted for 35.7% of all abortions and stillbirths.¹ Some umbilical twisting were normal, averaging 4 - 5 twists.² Excessive torsion occludes umbilical vessels and the urachus, resulting in decreased perfusion and fetal death. Umbilical edema, hemorrhage, thrombosis and fluid-filled sacculation were commonly observed along umbilical cord.^{1,3} Long umbilical cords predisposed cords for excessive torsion, with cords > 84 cm having a higher risk.^{1,4} Etiology of abnormally long umbilical cords is unknown.² Mares that aborted due to umbilical cord torsion had successful subsequent pregnancies.² Fetuses were not expelled immediately after death and generally had autolytic changes.³ Abortions were most common at 6 - 8 months.² Infectious causes should be ruled out in all abortions to reduce transmission. In this case, fetus was tested for Equine Herpes Virus 1 and *Leptospira interrogans* using PCR and both were negative. A needle aspirate of lungs had only mucus. Allantois was unremarkable. Diagnosis of abortion secondary to umbilical cord torsion was confirmed.

Keywords: Equine, mare, abortion, umbilical cord torsion, noninfectious

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Left testicular rupture in a Red Angus bull

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A 16 month old, Red Angus, bull presented with a history of bilateral scrotal swelling. On presentation, his scrotum was swollen and firm on palpation. There was a line of demarcation at the ventral aspect of scrotum, suggestive of trauma. Pain was elicited on palpation of scrotum. Ultrasonography of scrotum revealed a loss of normal homogenous echogenicity of testicular parenchyma and disruption of testicular architecture. No blood flow was detectable on the affected side with Doppler examination. Inguinal herniation was ruled out by ultrasonography of scrotum and transrectal palpation; however, diagnosis remained undetermined until surgery was performed. Scrotal enlargement most commonly results from fluid accumulation in the vaginal cavity.¹ Typically this fluid can be readily observed by ultrasonography, but was not apparent in this case. Traumatic rupture of testis has been reported to occur occasionally and can be diagnosed on palpation and confirmed by ultrasonography. Unilateral castration was performed to conserve the function of the unaffected testis. It was readily apparent after incising the scrotum that the bull had suffered a traumatic rupture of left testis. There was a large blood clot contained within the scrotum and disruption of vaginal tunics and tunica albuginea. Bull recovered uneventfully from surgery and was discharged with follow up appointments for suture removal and 90 days postsurgery for Breeding Soundness Examination (BSE). Following unilateral castration, remaining testis hypertrophied and produced ~ 75% of normal sperm capacity.² Although these bulls have been successfully used for breeding, they will not pass a BSE with 1 testis. This bull returned to service and was maintained as a breeding bull for several years after his procedure.

Keywords: Angus, bull, testis, hemicastration

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Successful treatment of ascending placentitis in a Thoroughbred mare

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Ascending placentitis in mares is 1 of the leading causes of late-term abortions, premature delivery, and neonate morbidity and mortality.¹ A 5 year old nulliparous Thoroughbred recipient mare presented on the 306th day of pregnancy for vulvar discharge without mammary development. Fetal viability was confirmed by transrectal and transabdominal ultrasonography. An increase in the combined thickness of the uterus and placenta (CTUP [17 mm]), was detected via transrectal ultrasonographic examination, although no discharge was evident on vaginal speculum examination. Based on examination findings, treatment for ascending placentitis was initiated using an established treatment protocol.² Antimicrobial (24 mg/kg oral sulfadiazine and trimethoprim twice daily), antiinflammatory (8.6 mg/kg oral pentoxifylline twice daily), and tocolytic (0.088 mg/kg of oral altrenogest daily) agents were given. In addition, 10 mg of estradiol cypionate (IM, once every 3 days, 3 doses total) was given, as it had improved clinical outcome of ascending placentitis cases.³ A decrease in CTUP was observed on transrectal ultrasonographic examination 8 days after initial presentation. However, all measurements did not return to normal limits; therefore, treatment had to be continued. All measurements of CTUP were within normal limits on transrectal ultrasonographic examination 16 days after initial presentation and the ascending placentitis was considered resolved. Transrectal and transabdominal ultrasonographic examination continued throughout pregnancy to monitor fetal health and screen for recurrent ascending placentitis. On 350th day of pregnancy, the mare delivered a healthy colt and grossly normal fetal membranes. The colt stood and nursed without assistance < 2 hours after parturition. Histopathological examination of fetal membranes demonstrated no evidence of placentitis. This case demonstrated that successful outcomes are possible in cases of ascending placentitis with early detection and aggressive treatment.

Keywords: Mare, ascending placentitis, parturition

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Anasarca in a neonatal bulldog puppy, secondary to a ventricular septal defect

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A 2 year old primiparous English Bulldog presented for fetal monitoring prior to elective cesarean section. Bitch was bred via transcervical insemination and her singleton pregnancy was confirmed via transabdominal ultrasonography and radiographs. Due to risk of primary uterine inertia and fetal oversize with 1 fetus, and due to being a brachycephalic breed, a cesarean section was scheduled. First fetal monitoring was performed at 64 days post LH peak; at this visit, bitch had colostrum present within caudal mammary glands, and her progesterone concentration was 6.32 ng/ml. On ultrasonography, although the fetal heart rate was normal (226 - 232 bpm), fetus appeared to have pleural effusion and subcutaneous edema, consistent with an anasarca puppy. Cesarean section was performed on the following day. A single pup with severe subcutaneous edema (confirmed ultrasonography findings) was removed. Euthanasia was elected due to poor prognosis and necropsy followed. Gross examination revealed a ventricular septal defect, a finding that has been associated with hydrops fetalis in human and veterinary species.¹ Renal tubules and renal pelvis were mildly dilated, suggestive of urinary obstruction presumptively due to severe anasarca. On histologic examination, hepatocellular necrosis and congestion of various organs were noticed (lesions secondary to decreased left-sided cardiac output). In summary, ultrasonography was diagnostic (anasarca fetalis and pleural effusion) and findings enabled us to decide on a timely surgery.

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Ejaculatory failure in a halter stallion due to dorsal displacement of soft palate

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Exercise-induced dorsal displacement of soft palate (DDSP) commonly occurs in racehorses, characterized by decreased performance;¹ however, it has not been identified as a cause for failure of ejaculation. In May 2019, a 5 year old maiden American Quarter Horse halter stallion was presented for failure of ejaculation. During breeding soundness examination, stallion demonstrated normal libido and mounting behavior during repeated mounts on phantom, but never ejaculated. While mounted, stallion expressed labored breathing that owner had noticed during previous breeding attempts. Manual palpation of scrotum, accessory sex glands, and penis were normal. An echocardiogram and chemistry analysis for lactic acid, alkaline phosphatase, and creatine kinase were normal. Static endoscopy of larynx was normal, but dynamic endoscopy during stallion's mount on phantom revealed 50 - 70% of airway became blocked by DDSP. To correct this condition, a laryngeal tie-forward procedure was performed. Following 2-week postoperative recovery under owner's supervision, stallion was able to ejaculate normally and repeatedly. Ejaculation disorders in stallions were attributed to physical, musculoskeletal, psychogenic, or neurologic causes;² apparently, DDSP has not been reported before as a cause. Halter-type stallions with ejaculatory dysfunction have been observed to exhibit labored breathing during and following breeding and was assumed to be a "common" breed characteristic. Occurrence of DDSP is associated with exercise intolerance; however, exact etiology is unknown.¹ In this stallion, increased activity was associated with breeding-induced displacement of soft palate above epiglottis. As a result, stallion was unable to complete ejaculation, due to blockage of airway. Following surgical correction, stallion was able to ejaculate. Ejaculatory dysfunction in stallions can be difficult to assess; therefore, DDSP should be considered as an underlying cause, especially when abnormal respiratory sounds are heard during breeding.

Keywords: Halter stallion, ejaculatory failure, DDSP

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Priapism in a Quarter Horse gelding

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Priapism is an uncommon finding in domestic animals; however, it is reported in horses. It has multiple etiologies, including use of phenothiazine tranquilizers for routine procedures such as sheath cleaning and dentistry.¹ Priapism results in a selective engorgement of corpus cavernosum and corpus spongiosum that ultimately leads to venous occlusion.² Exact mechanism for initial failure of detumescence is not known, but ultimately, red blood cells become sickle shaped in response to increased partial pressure of CO₂.³ This deformation in red blood cells can lead to permanent obstruction of venous drainage. A 25 year old, Quarter Horse gelding was presented with paraphimosis (noticed 2 days prior to presentation) secondary to priapism. His teeth had been floated 1 week earlier, under acepromazine sedation. Initial efforts to address priapism included placement of 12 gauge trocar needles, 1 dorsally and 1 ventrally within corpus cavernosum for irrigation with heparinized saline. Egress fluid from ventral trocar needles were thick, dark, and serosanguineous. As flush continued, fluid consistency changed to thin, bright red, serosanguineous to sanguineous, suggesting intact arterial supply. Phenylephrine was also administered directly into corpus cavernosum to promote vasoconstriction and reduction of priapism. Complete detumescence was not achieved by flush or phenylephrine treatment. A vascular shunt was established from corpus cavernosum to corpus spongiosum to relieve venous obstruction. Two days after the procedure, there was minimal improvement. Humane euthanasia was performed due to quality of life and financial constraints. At necropsy, vascular shunt (communication between corpus spongiosum and corpus cavernosum) appeared intact. Chronicity of the disease might have prevented full resolution and recovery, despite creation of a functional vascular shunt.

Keywords: Equine, gelding, priapism, acepromazine, corpus cavernosum, corpus spongiosum

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Changes in milk pH, milk protein, and udder development in late-pregnant ewe and effects on lamb serum total protein concentrations

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Objective was to describe changes in milk pH, milk protein concentrations, and udder development in late-pregnant ewes. Ewes were synchronized, ram bred, and confirmed pregnant ($n = 18$) for potential cesarean section at 145 days of pregnancy (d 0). Udder score was assessed on days 22, 15, 10, 7, 4, 1, and 0 prepartum. Milk pH (digital pH meter) and protein concentrations (Brix refractometer) were monitored once daily from days 6 to 0 prepartum. Twelve ewes were selected for elective cesarean section and induced with 20 mg dexamethasone IM on day 1. At birth, lambs were assigned a vigor score (1 - 10) based on heart rate, respiratory rate, muscle tone, irritability reflex, and mucous membrane color. A total of 19 live and 3 dead lambs were delivered. Serum total protein (TP) was measured in lambs from 24 to 36 hours after birth. Statistical analyses were performed using R. Data on days prepartum and lamb TP were analyzed using ANOVA. Pearson's correlation analysis was used to determine associations. There was moderate correlation in days prepartum with both udder development ($r = 0.53$, $p < 0.001$) and milk pH ($r = 0.46$; $p < 0.001$), but no correlation with milk protein concentrations ($r = 0.11$, $p = 0.25$). Days prepartum did not differ between udder scores of 0 (no development: 14 ± 3.8 days) and 1 (minor development: 12 ± 1.2 days; $p = 0.96$), but did change for scores of 2 (moderate development: 6.6 ± 0.5 days) and 3 (full development: 3.4 ± 0.4 days; $p \leq 0.03$). Days prepartum differed when milk pH measured > 6.5 (4.4 ± 0.3 days), $6 - 6.5$ (2.1 ± 0.2 days), and < 6 (0 ± 0 days; $p < 0.001$). Lamb TP was not affected by milk protein concentrations at d 0 ($p = 0.96$), but tended to be affected by vigor score ($p < 0.06$). We concluded that udder score and milk pH may be useful for predicting parturition in ewes. Additionally, assessing neonate vigor at birth can help influence colostrum management decisions.

Keywords: Milk pH, milk protein, neonate vigor, parturition, udder development

Canine herpes virus in a breeding kennel of Wirehaired Dachshund

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A 3-year-old, Wirehaired Dachshund presented to Auburn University Veterinary Hospital 53 days postovulation for dystocia and delivery of premature stillborn puppies. Rapid slide agglutination testing (RSAT) was performed for suspicion of *Brucella canis* infection, but was negative. Seven days later, the patient's kennelmate was presented 57 days postovulation for abortion, and *Brucella* RSAT was again negative. Canine herpesvirus (CaHV-1) was then investigated as a possible causative agent of serial abortions. A vaginal swab from the second patient was subjected to direct fluorescent antibody testing. Results were positive for CaHV-1 antigen. Symptoms of CaHV-1 infection include abortion, stillbirth, and infertility in bitches, whereas CaHV-1 can also cause "fading puppy syndrome" in neonates. Client returned 2 days later with 4 additional pregnant dams for testing. Vaginal swabs from all females tested positive for CaHV-1. All bitches also possessed a low or negative titer to CaHV-1. Based on these results, future litters were considered 'at risk' for contracting CaHV-1 infection. Recommendations included increasing the temperature in the neonatal area and implementation of biosecurity. Pregnant dams were prescribed oral acyclovir (20 mg/kg), to be administered every 6 hours until puppies were 3 weeks old. Once whelped, puppies received oral acyclovir solution at 15 mg/kg until 3 weeks old. With 3 successful litters, 13 puppies were born and alive at 6 months. Owners revealed that a new stud dog had been imported from Germany approximately 1 month prior to the initial abortion. This case highlighted the importance and magnitude of CaHV-1 infection in breeding bitches and neonates. Though most adult dogs are not clinically affected by CaHV-1, the virus can rapidly result in widespread loss in a breeding kennel by compromising the health of serologically naïve dams and neonates. In the case of this kennel's CaHV-1 outbreak, a positive outcome was reached through proper biosecurity and veterinary intervention.

Keywords: Canine herpes, abortion, stud dog

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A retrospective analysis on determinants of litter size in a colony of working dogs

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Successful breeding and increased litter size are paramount to increasing working dogs' numbers for national security. We determined key factors affecting pregnancy and litter size in Canine Performance Sciences (CPS) breeding colony. Specific aims of this research were to elucidate factors that improved breeding outcomes in the colony. Data from CPS breeding population for 55 breeding cycles for 26 females over 5 years were analyzed. Factors analyzed were: age of dam at breeding, semen type (fresh, cooled-shipped, frozen), insemination method (vaginal AI, transcervical insemination [TCI], live cover), and total number of inseminations. Multiple regression model was used. Above factors influenced ($p < 0.001$) and explained 57% of the variance in litter size. Addition of 1 TCI increased ($p = 0.005$) litter size. Addition of 1 live breeding, although not different ($p = 0.07$) had clinical relevance (increased litter size). Average litter size of a 30 month old dam using fresh semen AI was 5.45 puppies and cooled-shipped semen AI was 7 ± 1 puppies. Age of dams at breeding ranged from 13 - 88 months and there was no effect of age or parity on litter size. Results indicated that maximum litter size occurred when fresh semen was utilized, with cooled-shipped semen being equivalent. Use of frozen semen was least successful, resulting in negative effects on pregnancy rate in this population. Scrutiny of insemination methods revealed that addition of 1 live cover increased litter size by 2 puppies, whereas addition of 1 TCI increased litters by 3 puppies. In summary, incorporation of 1 live breeding and 1 TCI utilizing fresh semen increased average litter size in CPS breeding colony.

Keywords: Canine, semen, transcervical, litter, breeding

Foal born using intracytoplasmic sperm injection to a Friesian mare with unexplained subfertility

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Intracytoplasmic sperm injection (ICSI) is an advanced reproductive technology wherein oocytes are retrieved from tertiary follicles and injected with a single sperm to develop into an embryo in vitro. Unlike embryo transfer, ICSI affords versatility for mares with a suboptimum uterine environment, blocked oviducts, or at postmortem where the opportunity for natural fertilization is not possible. ICSI can also be used when limited numbers of poor-quality sperm are available.¹ This technique provides the possibility to obtain foals from mares that otherwise could not propagate their genetics. A 13 year old Friesian mare presented to the UC Davis Theriogenology service in May 2013 for breeding with cooled semen after several years of unexplained subfertility. Uterine cytology was taken at multiple intervals, with no clear evidence of endometritis. On ultrasonography, anechoic fluid was present in the uterus after all breeding attempts. Treatments included uterine lavages and oxytocin to enhance uterine clearance. In 2016, after several unsuccessful breeding cycles, laparoscopic application of prostaglandin E₂ in the oviducts was performed, with the suspicion that they could be blocked.² After the procedure, there were 2 more years of unsuccessful breeding attempts. In 2018, it was decided that this mare was an ideal candidate for oocyte retrieval and ICSI. In May 2018, transvaginal aspiration of oocytes was performed, where 15 follicles between 10 - 30 mm were aspirated from both ovaries. Of the 15 follicles aspirated, 9 oocytes were recovered. Four of these oocytes matured to the MII stage in vitro and underwent ICSI. Two embryos matured and were shipped as blastocysts to Colorado State University where they were implanted into 2 recipient mares. Both embryos survived until fetal heartbeats were detectable, with 1 pregnancy surviving to term. The 7 year old recipient mare was transported to UC Davis where she foaled successfully and without complications.

Keywords: Equine, intracytoplasmic sperm injection, mare subfertility

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Granulosa cell tumor and prolonged estrus in a Boxer bitch

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An 11 year old, multiparous Boxer bitch was presented to the Virginia-Maryland College of Veterinary Medicine Theriogenology Service for evaluation of persistent vulvar swelling and bloody vulvar discharge of 34 days duration. On physical examination, the vulva was markedly swollen, with a small amount of blood-tinged discharge. Vaginal cytology revealed 100% superficial epithelial cells, confirming a diagnosis of estrogen influence. Thorough history revealed no known exposure to exogenous estrogens. Abdominal ultrasonography was performed to identify potential structural abnormalities of the reproductive tract and/or adrenals which could be responsible for estrogen influence. Ultrasonography revealed a markedly thickened myometrium and endometrium, with unremarkable ovaries and adrenal glands. Furthermore, serum progesterone concentrations via chemiluminescence were 1.02 ng/ml. Based on the clinical findings, our differential diagnoses were microscopic disease of the ovary or exogenous estrogen exposure. Because the bitch had no history of exogenous estrogen exposure, microscopic ovarian disease, such as a granulosa cell tumor, was considered to be the most likely differential. Despite ovarian neoplasia being rather uncommon, granulosa cell tumors account for approximately half of all ovarian tumors in bitches.¹ It is not unusual for granulosa cell tumors to produce estrogen and therefore cause clinical signs consistent with a prolonged estrus. Definitive diagnosis of granulosa cell tumors relies on histopathology of ovaries. The owner elected not to pursue recommended ovariohysterectomy and instead elected humane euthanasia due to orthopedic comorbidities, with postmortem necropsy. Histopathology of a small (2 mm in diameter) round mass within an ovary revealed a well demarcated, proliferative mass made of tubules and small cysts lined with foamy basophilic cells, consistent with a granulosa cell tumor. This case demonstrated the importance of considering microscopic disease when presented with a bitch exhibiting prolonged estrus.

Keywords: Granulosa cell tumor, prolonged estrus, ovarian neoplasia, bitch

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Detection of *Tritrichomonas foetus* in a beef herd

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A herd of 150 Angus cows had transrectal palpation for pregnancy diagnosis at the end of April 2019. Eighty two percent of cows were determined to be pregnant. Eight nonpregnant cows had pyometra that was diagnosed by transrectal palpation and confirmed via transrectal ultrasonography. Pregnancies ranged from 25 to 110 days of age. Four Angus bulls were with the cows during a 120 day breeding season. Last year's weaning in the group was 77.57%. Smegma was collected from these 4 bulls via preputial scraping utilizing Pizzle Stick (Lane Manufacturing, Denver, CO). Samples were placed in Modified Diamonds Media and submitted to Bishop-Thompson-Sparks-Alabama State Diagnostic Laboratory and tested for *Tritrichomonas foetus* (*T. foetus*) DNA, via a reverse transcriptase polymerase chain reaction (RT-PCR) assay. Two of the 4 bulls tested positive for *T. foetus*, with strong positive cycle threshold (CT) values of 33.3 and 34.2. Two bulls that tested positive were sold to slaughter and 2 remaining bulls were retested via RT-PCR and were negative again. All cows with pyometra were sold for slaughter. Remainder of nonpregnant cows were treated IM 3 times with 5 ml of dinoprost tromethamine (Lutalyse) at 45 day intervals beginning in October 2019 to aid in clearing infection. These cows were then bred via AI in January 2020. The 2 positive bulls had been previously tested for *T. foetus* via RT-PCR and were negative in 2017 - 2018. Therefore, it was speculated that *T. foetus* entered the herd through replacements from another state. This case highlighted the importance of testing all bulls yearly for *T. foetus* when the herd is not a closed herd.

Keywords: Angus bulls, *Tritrichomonas foetus*, pyometra, smegma, preputial scarping, RT-PCR

Genetic causes of Golden Retriever congenital hypomyelinating polyneuropathy

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Myelin is an important component of both central nervous system (CNS) and peripheral nervous system (PNS) by surrounding nerve cells, protecting them and aiding in perpetuation of signal conduction. Myelin deficits can occur either by demyelination (myelin that initially formed properly and was then degraded) or by hypomyelination (myelin never correctly/fully formed at all). Two cases of littermate, full-sibling Golden Retriever (GR) puppies with very young onset (i.e. congenital) had neurological deficits.¹ Peripheral nerve biopsies revealed myelin sheath changes consistent with a predominantly hypomyelinating neuropathy. Uniquely, this syndrome affected only the PNS. Previously reported hypomyelination syndromes in veterinary patients had either exclusively involved the CNS, or both the CNS and the PNS together. We acquired DNA from 4 GRs presenting with clinical signs as reported;¹ nerve biopsies confirmed these dogs to have a congenital PNS hypomyelinating neuropathy. We performed whole genome sequencing on DNA from each of these 4 dogs. Three causative private variants were identified, each in an excellent functional candidate gene known to cause Charcot-Marie-Tooth disease in humans: a loss of donor splice site variant in 1 dog, a missense variant in 1 dog, and a premature stop codon shared by 2 dogs. These 3 variants were all absent from > 1,000 canine whole genome sequences of normal dogs, and from > 200 control GRs. While this condition is rare in the GR breed, breeders could use genetic tests for these variants to avoid producing affected puppies. In conclusion, theriogenology contains within its purview inherited congenital diseases. Four cases of congenital hereditary neurological disease in Golden Retrievers, wherein, using modern whole genome sequencing technology, we identified the likely causative mutation in each of them. Therefore, this is important for theriogenology service, as we can now offer each of these 3 variants as genetic tests to Golden Retriever breeders and prevent any more puppies born affected with this condition.

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Delayed uterine prolapse in a Thoroughbred mare with retained fetal membranes

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A 6 year old Thoroughbred mare was presented to Virginia-Maryland College of Veterinary Medicine's (VMCVM) Equine Field Service for evaluation of retained fetal membranes (RFM) after aborting twins the night before. Fetal membranes retention was observed by the owners, with no progression for 5 - 6 hours. Initial treatment included gentle manual traction of fetal membranes, administration of oxytocin and flunixin meglumine, and lavage of uterus. Despite expelling large amounts of fluid after lavage, there was no further progress in fetal membranes expulsion. Mare was given another oxytocin injection which caused continued myometrial contractions; however, there was no progress. Due to duration of retention, worsening of clinical signs (increased digital pulses and injected mucus membranes), and lack of improvement, mare was referred to the VMCVM Veterinary Teaching Hospital. She was resedated, and her uterus was lavaged again without dislodging fetal membranes. Mare was placed on IV fluids and broad-spectrum antimicrobials, and was under continued sedation and oxytocin treatment. At 8 hours after presentation, mare's uterus prolapsed (everted). Mare was given an epidural, uterus was rinsed with hypertonic saline and replaced. A Caslick's procedure was performed to aid in uterine retention. There was still a small amount of RFM observed after replacement. Hospitalization for treatment included twice-daily uterine lavages, IV fluids, oxytocin, antimicrobials, flunixin meglumine, and omeprazole. Mare was also treated with ice boots, vasodilators, and antiinflammatories to prevent laminitis. Mare was discharged 4 days later with no medications and did not experience any further complications. Uterine prolapse (eversion) is rare in mares, often fatal (from infection), and usually occurs immediately or within a few hours after parturition. Aggressive and prompt treatment of RFM in mares is critical. Regardless, as observed in this case, RFM may be considered a predisposing factor for delayed uterine prolapse.

Keywords: Equine, uterine prolapse, retained fetal membranes

Importance of breeding management in abnormal estrous cycles: a case study

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In the expanding small animal breeding industry, accurate estrus detection and ovulation timing are essential for breeding management. Many clinics rely on inhouse progesterone tests for convenient and efficient results. Despite widespread use of these tests, it is critical to monitor progesterone concentrations comprehensively due to potential abnormalities in the estrous cycles of individual patients. Such an anomalous cycle was diagnosed in Suzie, a 3.5 year old Fawn Chinese Pug, who was presented to Crestwood Veterinary Centre for artificial insemination. Suzie's baseline progesterone concentration on December 18, 2019 was 0.8 nmol/l (0.25 ng/ml) with concurrent clinical proestrus signs. Regular progesterone monitoring at 2 - 3 day intervals continued until December 27, 2019, where an increase to 4.0 nmol/l (1.26 ng/ml) indicated an impending luteinizing hormone (LH) surge. Despite this, on December 30, 2019, progesterone concentration decreased abruptly and was undetectable. Vaginal cytology had cornified cells confirming estrus. Suzie was bred on January 1 and 3 via artificial insemination with fresh semen; however, pregnancy was not achieved. A theriogenologist reviewed this case and suggested that Suzie's progesterone decrease after an apparent LH surge indicated she had not ovulated, and therefore should not have been bred. Her progesterone readings suggested an atypical estrous cycle, possibly a split heat or anovulatory cycle.¹ It was recommended to monitor Suzie's next cycle using serial examinations of serum progesterone and vaginal cytology, and to breed only after confirmation of ovulation via serum progesterone 2 - 3 days after the perceived LH surge. It was also suggested to induce ovulation using gonadotropin-releasing hormone or human chorionic gonadotropin in case of repeated anovulatory cycles. With advances in theriogenology, we are better able to understand dysfunctional or abnormal canine estrous cycles and to provide suitable advice to our clients in their management.

Keywords: Estrus, progesterone, canine, luteinizing hormone, artificial insemination, ovulation

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Sertoli cell tumor in an alpaca

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A 9 year old intact male alpaca was presented with a unilaterally enlarged right testis that the owner noticed after annual shearing. He had no previous breeding history, nor history of health or behavioral problems. Physical examination was normal except for visible unilateral scrotal enlargement. Scrotal palpation findings were, a large turgid right testis and a soft left testis, with normal epididymides. Palpation elicited no pain response and ruled out the presence of epididymitis, herniation, or testicular torsion. Testicular ultrasonography revealed a heterogeneous appearance of right testis parenchyma with diffuse multifocal areas of hyperechoic foci and several anechoic pockets. Left testis was comparatively homogenous in appearance with rare hyperechoic foci. At this point, differential diagnoses included neoplasia or orchitis. A bilateral elective castration was performed, since this alpaca was not intended for breeding. After castration, right testis measured 50 x 54 mm, with 2 cystic structures and a bulging cream-colored bulging structure grossly identified upon dissection. Left testis measured 37 x 32 mm with no gross abnormalities. Histopathology diagnosed a Sertoli cell tumor that completely replaced the stroma in the right testis and diffuse degeneration with no tumor infiltration in left testis. Spermatic cord was also free from tumor involvement. The alpaca recovered from castration uneventfully. Testicular neoplasia in camelids is rare. While Sertoli cell tumors have been reported in dogs, bulls, rams, cats, and horses, apparently, this is the first report in camelids. Sertoli cell tumors are characteristically hormonally active. While no symptoms were present in this case, degeneration of contralateral testis suggested that the tumor might have been secreting estrogen. Bilateral castration proved to be curative for this alpaca; however, since no metastasis was evident, unilateral castration might have preserved fertility, if there was an intention to use the animal for breeding.

Keywords: Sertoli cell tumor, testicular enlargement, testicular degeneration, alpaca

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Scrotal hydrocele in an Angus bull

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A 5 year old, Angus, bull presented with a history of left testicular swelling. Ultrasonography of scrotum revealed a normal appearing right testis and the presence of fluid containing strands of hyperechoic material surrounding left testis. No bowel was appreciated within the scrotum. Semen evaluation revealed decreased numbers of sperm with adequate motility. White blood cells were present on the semen slide and proximal droplets were seen within individual sperm. The fluid surrounding left testis indicated unilateral scrotal hydrocele. Hydrocele is an abnormal accumulation of serous or inflammatory fluid within the vaginal cavity of testis.¹ In mature bulls, hydrocele results from local inflammation or trauma.² Most commonly, scrotal hydroceles present with swelling in scrotum.³ Ultrasonographic examination reveals varying amounts of anechoic fluid surrounding the epididymis and testis with normal testicular echotexture.^{3,4} With fluid accumulation, testicular thermoregulation is disrupted, resulting in decreased semen quality.³ Treatment of this condition is directed at removing the inciting cause of fluid accumulation.³ In this case, a hemicastration of left testis was performed with the possibility of regaining reproductive potential. Cystorelin (GnRH) was administered to increase spermatogenesis in the remaining testis. At re-examination, appropriate postoperative healing was noted. A semen sample obtained via electro-ejaculation had 16% normal cells, 79% cells having primary abnormalities and 5% cells having secondary abnormalities. Other parameters of the breeding soundness examination were concluded to be within normal limits. The scrotal circumference was 29 cm. Prognosis for reproductive performance in bulls with unilateral hydrocele is good with prompt removal of affected testis.³ Since spermatogenesis can be impaired for 2 - 6 months following testicular insult, final recommendations regarding fertility should only be made after allowing adequate time for recovery.^{3,5}

Keywords: Hydrocele, bull, castration, BSE, ultrasonography

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Bilateral ovarian adenocarcinoma in a geriatric Shih Tzu

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A 15 year old female intact Shih Tzu was presented due to vaginal discharge of 4 day duration that had been recurring for over 1 year. She had a history of an open pyometra twice in the past year. Ovariohysterectomy had not been pursued, due to owner's concern for anesthetic risk. On presentation, the patient was moderately thin, had serosanguinous vaginal discharge, a swollen vulva, and a positive flagging response; physical examination was otherwise unremarkable. Vaginal cytology revealed primarily intermediate cells with few parabasal cells. Vaginal cultures revealed growth of *Enterococcus faecalis*, *Escherichia coli*, and *Staphylococcus pseudointermedius*. Progesterone concentration was 0.79 ng/ml. Transrectal ultrasonography revealed cystic structures on both ovaries. Followup examinations within next 2 weeks revealed similar findings on vaginal discharge and cytology, and progesterone concentrations rose slowly to 2.28 ng/ml. Due to the abnormal ovarian findings and potential for hyperestrogenism, ovariohysterectomy was pursued. Preoperative bloodwork revealed a mild leukocytosis characterized by a neutrophilia with a mild left shift. Histopathology of reproductive tract revealed bilateral ovarian adenocarcinoma, severe, diffuse, chronic cystic endometrial hyperplasia, and mild pyometra. Epithelial tumors, including ovarian adenomas and adenocarcinomas, were the most common primary ovarian tumor in dogs (46%), with adenocarcinomas making up 20% of canine ovarian tumors.¹ The patient recovered without incident and was discharged to the care of her owner. Followup examination 3 weeks postoperatively, including thoracic radiographs and abdominal ultrasonography, revealed no evidence of metastasis. This case presented a unique abnormal estrous cycle secondary to a neoplastic process in a geriatric bitch with an open pyometra.

Keywords: Ovarian adenocarcinoma, geriatric dog, hyperestrogenism

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Bilateral cystadenoma with a granulosa cell tumor in a Bernese mountain dog

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A 7 year old Bernese mountain dog presented for more than 5 weeks of persistent estrus and infertility. Her history consisted of multiple attempts of unsuccessful breeding, with and without progesterone timing, and she had never produced a litter. On presentation, the bitch had normal physiological parameters with the presence of a swollen vulva and vaginal bleeding. Vaginal cytology revealed 100% superficial cells, consistent with estrus. Serum progesterone was 0.671 ng/ml, also consistent with estrus. Transabdominal ultrasonography was elected to examine for the presence of follicular activity or any abnormalities. The right uterine horn presented with a small single cyst. The right ovary was larger and with a lobulated appearance and a questionable small mass. In addition, the right ovary had increased blood flow on Doppler compared to the left ovary. Due to her advanced age, history of infertility and ultrasonographic findings, an ovariohysterectomy was performed. Morphological diagnosis on histopathology was bilateral papillary cystadenoma, a granulosa cell tumor (GCT) on the right side and aggregates of small cells, indicative of an early dysplastic change over a GCT on the left side. According to an analysis of studies of canine ovarian tumors from 1960 - 2004, granulosa cell tumors accounted for ~ 25% of all ovarian tumors.¹ A case that had a history of infertility and presented with clinical signs of estrus was diagnosed to have bilateral papillary cystadenoma and a unilateral GCT.

Keywords: Bilateral cystadenoma, granulosa cell tumor, infertility

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A third-degree rectovaginal tear in a primiparous mare

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A 10 year old Quarter Horse mare was presented for rectovaginal tear and fistula. She had foaled unattended. The large foal was found dead but appeared grossly normal. A tear was present from the mare's vulva through her perineal body and extended cranially 6 inches. The mare was medically managed with flunixin meglumine and femycin and hydrotherapy to reduce irritation from fecal contamination. The owner was advised to keep her on a soft stool diet and to continue hydrotherapy for 6 weeks prior to surgical reconstruction. Two months after the initial visit, the mare was reevaluated for surgical correction of the rectovaginal tear. On rectal palpation, a 5 x 5 inch fistula was noted and the rectal sphincter was intact. Fecal contamination of the vagina was present. Surgical correction for this case was performed in 2 phases. In the first phase, the rectal sphincter was split and the fistula was converted to a grade 3 rectovaginal tear. In phase 2, the perineal body and anal sphincter were repaired. Due to the degree of fibrosis from the severity of her injury, additional foaling was not recommended. Third-degree rectovaginal tears occur predominantly in primiparous animals, with the mare being over represented, due to the explosive nature of their parturition.¹ Third-degree rectovaginal tears are a complete communication between the vestibule and the rectum. Correction is important to either restore breeding function or to provide a better cosmetic appearance.

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Cervical adenoma and excisional biopsy in a spayed Labrador retriever bitch
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A 7 year old (42 kg), nulliparous, spayed female Labrador Retriever was presented following referral to the Virginia Maryland College of Veterinary Medicine for evaluation of chronic, intermittent, bloody vaginal discharge and acute tissue herniation from the vulva. Physical examination revealed a hemorrhagic, multilobulated, irregularly shaped mass protruding from the vulva. A digital vaginal examination revealed the presence of a firm, prominent, pedunculated mass originating from a distinct stalk. An impression smear had a monomorphic population of large, undifferentiated round cells with anisocytosis, anisokaryosis, and coarse chromatin. Following sedation with intravenous dexmedetomidine (7 µg/kg) and butorphanol (0.3 mg/kg) and administration of a lumbosacral epidural (0.25 mg/kg of 0.75% preservative free bupivacaine), digital vaginal examination revealed 2 pedunculated masses. The caudal mass originated from ventral vaginal mucosa located 5 mm cranial to the urethral orifice, and the cranial mass was located dorsolaterally, 1.5 cm cranial to the first. The owner elected excisional biopsy of both masses for histopathology performed with a vessel sealing device (Ligasure) over an episiotomy with complete excision. Although the caudal mass was easily exteriorized and excised, the cranial mass was more friable and tore during attempted exteriorization, leaving a remnant within the cranial vagina. Minimal vaginal bleeding was observed postbiopsy. The dog was discharged with carprofen (2.2 mg/kg PO, every 12 hours) and trazodone (2.4 mg/kg, PO, every 12 hours) awaiting histopathology. Histopathology revealed a well-differentiated simple cuboidal and columnar epithelial population of cells, typical of the cervix, with secretory mucin-rich cystic glandular lesions consistent with cervical adenoma. Vaginoscopy was repeated at 1 month postbiopsy. The remaining mass had not changed in size or appearance; however, the previous clinical signs had resolved. While benign epithelial tumors of the vaginal mucosa have been documented, this case presents the first reported benign epithelial tumor of the cervix, as well as a minimally invasive technique for treatment.

Keywords: Cervical adenoma, Ligasure, canine, vaginal bleeding

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