Society for Theriogenology
Veterinarians Dedicated to Animal Reproduction

Clinical Theriogenology

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

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Clinical Theriogenology

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Clinical Theriogenology

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

Mission Statement

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

Scope of the Journal

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

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

Publication Schedule

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

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. The manuscript must be in English (American spellings), and follow the Uniform Requirements for Manuscripts Submitted to Biomedical Journals (http://www.icmje.org). The following guidelines are applicable:

- Manuscripts should be submitted to the editorial office as an e-mail attachment or on a CD compatible with Microsoft Word. The disk should be accompanied by a hard copy of the manuscript. Submit manuscripts to:

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  Editor, *Clinical Theriogenology*
  W-203 Veterinary Medicine Building
  University of Missouri
  Columbia, MO 65211
  573-884-6774 (voice)
  573-884-5044 (fax)
  or to the following e-mail addresses:
  youngquistr@missouri.edu
  clinicaltherio@therio.org
  therioeditor@therio.org

- All pages are to be double-spaced
- Font: Times New Roman; size 12
- Left-justified
- 1" margins at the top, bottom, and sides of each page
- All pages (including the title page) are to be numbered consecutively

- All lines of the manuscript should be numbered consecutively

The general format for scientific manuscripts is as follows:

- Title page: Contains the title of the paper and the first and last names of each of the authors; middle initials are optional. Specify the corresponding author and her/his contact information (mailing address, telephone and fax numbers, email address). Do not list academic degrees or specialty board certification. State the sources of funding (if any) and any meetings at which the data were presented.
- Abstract and keywords: The abstract should capture the essence of the paper and should be limited to 250 words or fewer. The term "Keywords" is typed in bold font followed by a colon followed by up to six key words separated by commas.
- Text: Begin the text on a separate page and divide it into the traditional sections of a scientific paper, viz. introduction, materials and methods, results, discussion, and conclusion.
- References: Only the most pertinent papers should be cited. References should be cited in consecutive order when first mentioned in the text, designated by a superscript number placed after all punctuation marks. The Vancouver style of citation is to be used with the exception that only the first three authors of multi-authored papers are listed; if there are four or more authors, list the first three, followed by et al. For examples, please consult: http://www.nlm.nih.gov/bsd/uniform_requirements.html Titles of journals are to be abbreviated in the style of Index Medicus. For assistance in locating the proper abbreviation for a scientific journal, see the following websites: http://home.ncifcrf.gov/research/bja - Biological Journals and Abbreviations. PubMed also has journal abbreviations available at http://www.ncbi.nlm.nih.gov/sites/entrez?db=journals Type the name of the journal into the search box and select "go". The next screen shows information about the journal including the proper abbreviation. One word titles are not abbreviated. Unpublished observations and personal communications should not be used as references. Articles listed as "in press" must have been accepted for publication. Authors are responsible for insuring the accuracy of all citations listed; citations must be verified against the original publications.

  Examples:
  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 three 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 (personal author)
  Johnson SD, Kustritz MVR, Olson PNS: Canine and feline theriogenology. Philadelphia: Saunders; 2001. p. 7.
  Book (edited, multi-author)
  Woods GL, Hallowell AL: Management of twin embryos and twin fetuses in the mare. In: McKinnon AO, Voss JL, editors. Equine reproduction. Philadelphia: Lea and Febiger; 1993. p. 532.
  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.

- Units of measurement: Measurements should be listed in Système Internationale (SI) units with non-SI units listed in parentheses if they are needed for clarification.

- Abbreviations: Abbreviations other than standard units of measurement should not be used excessively but if necessary, the abbreviation must be defined at first use in the manuscript. Do not use abbreviations in the title or in the abstract. Abbreviations are used exclusively subsequent to being defined except at the beginning of a sentence.
- Tables: Data can be summarized in well-designed tables prepared with the table function of Microsoft Word. Each table should be on a separate page with the legend typed above the table. Tables are numbered consecutively as they appear in the text.
- Numerals: Spell out numbers one through ten and note 11 onward as numerals. Write out numbers if they appear as the first word in a sentence.
- Illustrations: Illustrations must not be embedded in the text. Figures are to be cited consecutively by number (Fig.1, Fig. 2, etc). All images and text are sent to the printer in digital format. Acceptable formats are .jpeg and .tiff. Minimum acceptable resolution is 300 pixels/inch for halftones and 1,200 pixels per inch for line art. Black and white illustrations are reproduced at no charge but the cost of including color plates will be billed to the author.
- Acknowledgements: Persons who make substantial contributions to the content of the article but who do not qualify as authors should be acknowledged. Authors must provide written permission from all persons who are acknowledged.
- Sources and manufacturers: Only generic names of drugs, chemicals, test kits, and equipment should be used in the text followed in parentheses by the tradename, supplier's name, and supplier's address (city, state [country if not in the United States]). For example: The cow was treated with 100 mcg gonadorelin hydrochloride im (Factrel™, Fort Dodge Animal Health, Ft. Dodge, IA).
- Manuscripts are initially reviewed by the editor and those that meet the requirements for publication are submitted to at least two reviewers. Authors are invited to suggest potential reviewers in their initial submission. Manuscripts are prepared for publication in the order in which they pass the peer review process

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

Instructions to Authors are subject to revision to meet the technical standards imposed by the printer and are to be reviewed annually and revised if necessary by the Editorial Board.

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From the President of the American College of Theriogenologists

It has been a very good year for Theriogenology. Many ACTs volunteered to serve our College for different positions on the board. On behalf of the Executive Board and the ACT, I would like to thank those colleagues who agreed to compete for the various open positions. Special thanks to Claire Card and Chuck Estill respectively, for convincing Theresa Burns and Fred Lehman to consider serving the College. I thank Gary Althouse, Bruce Eilts, Lloyd Kloppe, Richard Linhart, and Carlos Pinto for agreeing to serve when I approached them. Thanks to those members who returned the ballots. The officers chosen to serve on the Executive Board will be introduced to the members at the Milwaukee business meeting.

My appreciation goes to the members of our Executive Board for helping us to shape the future of different programs. I have been immensely helped by my colleagues from slipping. The Executive Board thanks Charles Franz and the management staff for their help in conducting the business of the College to members' satisfaction. The Executive Board is looking forward to its meetings in Milwaukee.

It is appropriate to recognize the help rendered by Jane Barber and Steve Brinsko for organizing an event to recognize the ACT charter diplomates at the Milwaukee meeting. Thanks are in order to the Board of Directors of the Society for Theriogenology and the Theriogenology Foundation for providing additional financial support for this event.

I trust that the members had a chance to read in the Spring Newsletter regarding the back-ground information and the contribution made to Theriogenology by two of our colleagues. The College congratulates Cheryl Lopate and Ahmed Tibary for winning the awards and appreciates these two caring diplomates for their distinguished service to our discipline. I do thank their ACT mentors and their institutions for recognizing their talents and giving them an opportunity. The College should be especially thankful to Ahmed and Cheryl for preserving the rights of theriogenologists to perform C-sections in teaching hospitals. Please join me in wishing them well in their future endeavors for Theriogenology.

The College is grateful to Bob Youngquist for his careful nurturing of our official journal 'Clinical Theriogenology'. Many of our members are contributing to the growth of the journal by submitting sound clinical and review papers. In this regard, members of the Executive Board are setting a good example for others to follow, thanks to Steve Brinsko and Ram Kasimanickam.

It has been a rewarding experience for me to serve our College in different capacities for the past 8 years. It is appropriate to recall what the first president mentioned with pride the uniqueness of our discipline – 'substantial internationalism'. David Bartlett rightfully said[1] "Theriogenology has been and is being greatly enriched by graduates of veterinary schools from other than those in North America." I am glad that I am part of an organization that strives very hard to provide opportunities for individuals with diverse backgrounds. Let me thank my able mentors, Bill Bosu, Joe Gains, and Cynthia Smith for investing in me. In closing, let me quote David Bartlett[2] regarding our role to the society which incidentally drives our destiny. "Clearly, demand for and utilization of Theriogenology by animal owners is profit motivated; humaneness, sentiment, or aesthetics are seldom involved. Ultimately, whether the future level of Theriogenology rises or falls in academia, research, and veterinary practice seems destined to be determined by economic values, as perceived and consequently demanded by the animal industries."

1. Bartlett DE. Theriogenology: from concept to actuality. Theriogenology 1985;24:131-45.
2. Bartlett DE. Factors influencing the evolution of theriogenology. J Am Vet Med Assoc 1989;195:51-5.

Augustine Peter

Future Theriogenology Conference Dates

Sponsored by
The Society for Theriogenology
and
The American College of Theriogenologists

The Conference Planning Committee is developing outstanding programs!

MAKE PLANS NOW TO JOIN US AT
THE FOLLOWING LOCATIONS:

2012
August 21-August 25
Baltimore, Maryland

2013
August 6-August 10
Louisville, Kentucky

2014
August 5-August 9
Portland, Oregon

Proceedings of the Annual Conference of the Society for Theriogenology

August 9 to 13, 2011

Milwaukee, Wisconsin, USA

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Species Abstracts

Equine

Manuscripts from the pre- and post-conference symposia (Theriogenology Educators' Forum, Small Ruminant Symposium, and National Symposium for Dairy Farmers) and conference manuscripts not received in time to be included in the Proceedings issue will be published in subsequent issues of *Clinical Theriogenology*.

The 2011 Bartlett Address
The education of a theriogenologist

Dwight F. Wolfe
Department of Clinical Sciences, College of Veterinary Medicine, Auburn University, Auburn, AL

"And Then There Was Serependity". These words were the first slide in Dr. Ana Montes Adams' seminar in preparation for defense of her Master of Science degree at Auburn University in 1994. I believe that statement describes how I came to be standing before you today.

I begin by extending my most heartfelt appreciation to the Society for Theriogenology and the American College of Theriogenologists for this honor you bestow on me. The invitation to present this lecture in honor of Dr. David E. Bartlett is a deeply humbling experience. With humility I graciously accept this award for all the mentors, students, residents and colleagues whose efforts have accomplished what this recognition was intended. I am especially fortunate to share the podium today with Dr. Bartlett, the gentleman who invented the term "Theriogenology" that brings us all together. I especially thank my family who believed in me and supported many long hours in my chosen profession, often neglectful of family activities.

I followed a very circuitous and perhaps non-traditional route to veterinary medicine. I was born in Memphis, Tennessee and my father worked for a railroad. In that job he was often transferred such that I attended 13 schools in five states by the time I graduated from high school. We never had a pet and I never lived on a farm although an uncle who farmed in Mississippi kept a horse for me and allowed me to spend much of my summers there. After graduation from high school I worked in a steel fabrication plant in Indiana then moved back to Memphis where I worked for a company that built railroad crossings and switches, then a sheet metal company that made roof flashings and water supply lines for sinks and toilets and later loaded and unloaded trucks for a freight line. I originally started college to become an architect then later changed to pre-veterinary medicine. I was a part-time student for many years and finished my B.S. degree in Animal Sciences at the University of Tennessee 10 years after graduation from high school. I was then fortunate to be accepted into the veterinary college at Auburn University where I graduated at 32 years of age as a "B" student.

So how does a vagabond city boy become a Food Animal Theriogenologist? Marie Dressler, Canadian actress, is quoted as saying "Never one thing and seldom one person can make for success. It takes a number of them merging into one perfect whole." By no measure am I perfect but many people educated me along the journey. I saw my mare deliver a foal in the moonlight by sneaking out of my uncle's house one spring night to see if the foal had been born yet. I developed an interest in reproductive physiology while at the University of Tennessee. During the veterinary curriculum at Auburn I was fortunate to be taught by theriogenologists Drs. Don Walker, Bob Hudson, and Ram Purohit, by equine surgeon Dr. John Vaughan, and by Dr. John Winkler, dairy veterinarian with excellent skills in bovine theriogenology. Each of these men, along with many others helped mold my interests in reproduction and clinical practice.

Just before I graduated from veterinary college in 1977 I served a 12 week preceptorship with Dr. Charlie Davis in Monte Vista, Colorado. Dr. Davis mentored me in how to become a veterinary practitioner in a ranch setting. Calving season in ranch country was a busy time and I left there with a zest for obstetrics and reproduction. After graduation I became an associate in a five veterinarian mixed practice in Jefferson City, TN. This was a typical mixed practice in that we covered too much territory and worked too many hours but I absolutely loved the work and the challenges. After three years I returned to Auburn for a graduate program and residency with the intent of returning to private practice. By this time theriogenologists Dr. Bob Carson, Dr. Gatz Riddell and Dr. David McClary were on staff. Drs. Hudson, Walker, Carson, Purohit and Dwayne Beckett guided my residency and graduate research. Upon completion of the residency

Dr. Walker offered me a faculty position in the Food Animal Section at Auburn and I have had an extremely rewarding career in that role.

You may recognize many of the names just listed. Dr. Bob Hudson, Dr. Don Walker and Dr. Bob Carson each received the Bartlett Award. Dr. Dwayne Beckett and Dr. Ram Purohit worked with Dr. Walker and others elucidating the mechanism of erection and penile corpus cavernosal pressures in the bull. Dr. Purohit is internationally acclaimed in thermography. Dr. J.T. Vaughan is a highly respected equine surgeon and co-authored the text Bovine and Equine Urogenital Surgery with Dr. Don Walker, for many years the premiere reference in its field.

Through the Society for Theriogenology and the American College of Theriogenologists I have developed friendships with colleagues in many parts of the country. We renew friendships at the annual conference and regularly communicate on cases, keep up with colleagues and on developments in our profession. A prime example of relationships that develop through these organizations is that on March 10, 2011, Dr. Larry Rice, now retired for several years, called to congratulate me on this award. I had the privilege to call Dr. Rice and notify him he was the recipient of this award for 2006. I first met Dr. Rice during the ACT practical exam in Nashville, TN, 1983. I nervously sat down across the table from him as he asked me to describe freezing bull semen. I am privileged to call him, like so many previous recipients of this award as friends and colleagues. They have shaped my career in innumerable ways.

So what does being a Theriogenologist mean to me today? It means I have been taught the skills that allow me to help animals and their owners in innumerable ways. Most of my day to day clinical activities are not what one would consider classical Theriogenology. Yes, I evaluate bulls for breeding soundness and examine females for suitability for breeding and for pregnancy. I take part in obstetrical management of females and with care of neonates. I perform restorative surgery on bulls and females that often allows them to continue their productive lives. I participate in research that focuses on basic and applied animal reproduction and diseases. Who among us doesn't feel a sense of accomplishment when we palpate or ultrasound the pregnancy or deliver the calf or foal or lamb resulting from the embryo we collected from a donor that we managed then harvested, graded and transferred into a recipient that we managed to ovulate synchronously with the donor? Of course we all do. Or resuscitate the litter of puppies or calf that we deliver by cesarean section knowing that the neonate and possibly the dam would have died without our help. Of course we all do. Or perform reconstructive urogenital surgery on a sire or dam who later successfully breeds? Of course we all do.

We now use sexed semen, DNA testing for disease susceptibility and production traits, ultrasound, cryopreservation and vitrification of embryos, in-vitro fertilization and cloning as well as develop transgenic animals. We have numerous quick tests for pregnancy status, hormone levels and disease diagnostics. Today we have the best vaccines, antibiotics, hormone products and protocols in history but still the basics of animal husbandry and well-being are the foundation for fertility and reproduction.

Even with all the wonderful technological advancements, the foundation of our clinical skills still lies with our ability to instill sufficient confidence in the client to allow us to obtain a reliable history and to be observant and perform a thorough physical examination. Auburn is fortunate to have 15 theriogenologists on faculty. We are veterinarians first, then theriogenologists. Technology and science do not replace the personal touch.

I am blessed to have taught nearly 3000 veterinary students as well as assisted with the education of numerous residents in theriogenology, large animal medicine, and large animal surgery. For several years I have taught a class in Herd Health Maintenance in the Department of Animal Science at Auburn. A significant portion of that class is devoted to animal reproduction and obstetrics. A few of those students go on to become veterinarians but the majority fill jobs in animal agriculture or other animal health fields. I believe we have a strong obligation to educate people engaged in animal production and to the public on the role veterinarians fulfill in ensuring animal well-being and in ensuring a safe and abundant food supply.

Never lose sight of the fact that we have a personal relationship with the client. We are extremely fortunate to be able to better the lives of people and their animals. Perhaps Eli Lilly said it best in 1893, "Foolish indeed is the business organization that measures its success solely with a profit yardstick and ignores its most valuable assets: the faith and good will of those whom it seeks to serve, and the faith and loyalty of those who are dependent upon it for happiness."

For production animals we can enhance the economic health of the farm and the owner through our services. How do we accomplish this? We are first and foremost veterinarians with a holistic approach to the herd, or flock as well as for the companion animal. We develop biosecurity plans and provide oversight for nutrition, housing, transportation and preventive health measures.

From the animal perspective we enhance their quality of life by applying our knowledge of breeding management, pregnancy diagnosis, obstetrics and post-partum care and to ensure that males are well-developed and satisfactory breeders and that females become pregnant in timely fashion to meet the production goals or the goals for the competitive athlete or family pet.

We determine pregnancy early in the fetal stage so that females are not unduly repeatedly exposed to breeding or maybe so that they do not carry a defective fetus to term. Early determination of pregnancy also enhances the accuracy with which we can predict time of parturition so that appropriate assistance can be available if needed.

The charter members of the Rocky Mountain Society for the Study of Breeding Soundness in Bulls, our parent organization, were focused on finding scientific solutions for issues of animal well-being and the welfare of their owners. The Society for Theriogenology still focuses on that goal. We are blessed to be members of a profession and organizations that are rich with mentors. Our members merge creativity and enthusiasm to form the synergy so vital for the future. The SFT and ACT are vibrant examples of the advice of Peter Drucker who is quoted as saying, "The best way to predict the future is to create it." We grow from our experiences and with the help of our colleagues. Many of us have chosen careers we would never have imagined and as I firmly believe the adage that "The key to a successful life is how you handle Plan B." I believe that is the key to happiness in our lives if we take advantage of our talents and beliefs. I challenge each of you to become involved and help these organizations and our clients to continue to prosper.

In closing, many people contributed to my career and I thank each of you who molded me into the person who stands at the podium today. I thank the members of SFT and ACT who are always helpful and forward thinking to advance our discipline. I thank the practitioners who call to consult on cases. I thank the colleagues whom I work with daily in the teaching hospital. I thank the clients who graciously allow me to assist their animals. I thank the students and residents who challenge me to answer questions and often to think outside the box. And finally, thank you board members and officers for sharing your time, your talents and friendship with me and for continuing to grow and improve the organizations.

Information searching techniques and staying current for the veterinarian

André J. Nault
Veterinary Medical Library and Veterinary Clinical Sciences, College of Veterinary Medicine, University of Minnesota, St. Paul, MN

This presentation will cover three main topics: 1) how to find quality, evidence-based veterinary information, 2) how to get full-text copies of this information or journal articles, and 3) how to stay current in the field of theriogenology with so little free time.

Free scholarly indexes

PubMed/MEDLINE

http://pubmed.gov

PubMed is a free search engine accessing the MEDLINE database of citations - around 20 million currently. It indexes over 5000 science journals, including 90-100 veterinary journals, and provides abstracts and some full-text articles on life sciences and biomedical topics. Approximately half of all veterinary journals are indexed in MEDLINE. The United States' National Library of Medicine (NLM) at the National Institutes of Health (NIH) maintains PubMed.

The scope of MEDLINE includes diverse topics such as microbiology, delivery of health care, nutrition, pharmacology, and environmental health. Categories include everything from anatomy, organisms, psychology to the physical sciences.

MEDLINE is a very high quality database, but one disadvantage is that it does NOT contain very much foreign materials (90% are English), books or gray materials (conferences, symposiums, etc).

Keyword searching works well in PubMed, and it will automatically try to map search terms with MeSH (Medical Subject Heading) terms in MEDLINE. MeSH can be viewed as a controlled vocabulary that allows much more controlled and precise searching. Performing a MeSH search instead of a keyword search will generally result in more quality results. Several tutorials are available on MeSH searching here: http://www.ncbi.nlm.nih.gov/mesh and here: http://www.nlm.nih.gov/bsd/disted/pubmedtutorial/

An example of the MeSH tree for "Assisted reproductive techniques", defined as: Clinical and laboratory techniques used to enhance fertility in humans and animals.

All MeSH Categories
- Analytical, Diagnostic and Therapeutic Techniques and Equipment Category
  - Therapeutics
    - Reproductive Techniques
      - **Reproductive Techniques, Assisted**
        - Embryo Transfer
          - Single Embryo Transfer
        - Fertilization in Vitro
          - Sperm Injections, Intracytoplasmic
        - Gamete Intrafallopian Transfer
        - Insemination, Artificial
          - Insemination, Artificial, Heterologous
          - Insemination, Artificial, Homologous
        - Oocyte Donation
        - Oocyte Retrieval
        - Ovulation Induction
          - Superovulation
        - Posthumous Conception
        - Sperm Retrieval
        - Zygote Intrafallopian Transfer

Compare this with the MeSH tree for "Reproductive Techniques", defined as: Methods pertaining to the generation of new individuals, including techniques used in selective BREEDING, cloning (CLONING, ORGANISM), and assisted reproduction.

All MeSH Categories
Analytical, Diagnostic and Therapeutic Techniques and Equipment Category
Investigative Techniques
Reproductive Techniques
Breeding
Estrus Detection
Estrus Synchronization
Hybridization, Genetic
Inbreeding
Cloning, Organism
Embryo Disposition
Fallopian Tube Patency Tests
Nuclear Transfer Techniques
Ovulation Detection
Ovulation Prediction
Reproductive Techniques, Assisted
Embryo Transfer +
Fertilization in Vitro +
Gamete Intrafallopian Transfer
Insemination, Artificial +
Oocyte Donation
Oocyte Retrieval
Ovulation Induction +
Posthumous Conception
Sperm Retrieval
Zygote Intrafallopian Transfer

Of course, theriogenology covers many other areas, and other MeSH headings examples include:
Hysterectomy/veterinary
Ovariectomy/veterinary
Sperm Count/veterinary
Cattle/embryology

MeSH searches also offer the option to limit results to specific subheadings, such as veterinary, mortality, economics, instrumentation, etc. Limits can also be used to restrict results to articles offering free full-text, or to specific article types. Some of the different article types that can be selected for under the limits tab include:

Review articles. These review the current literature on a particular topic. The Veterinary Clinics of North America from Elsevier is one example of a journal containing review articles. If you use PubMed, notice the review tab on the right of the display window in order to select just review articles during a search.

Meta-analysis. These combine the results of several studies that address a set of related research hypotheses.

Randomized control trial. An experiment where subjects are randomly allocated to receive one or other of the alternative treatments under study.

Controlled clinical trial. A clinical trial that includes a control group but no or inadequate methods of randomization.

Case studies. Descriptions of a major healthcare intervention, usually from a public or herd-health perspective. Case study articles should include a rigorous assessment of the processes and impact of the intervention as well as recommendations for future interventions.

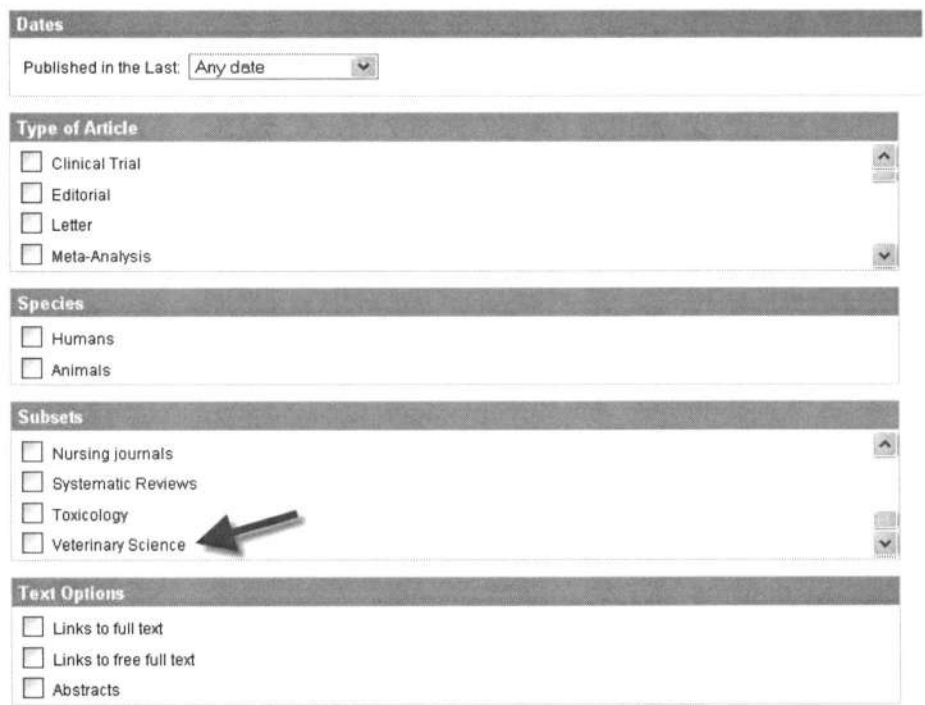
Case report. Typically a report on a single individual, these are reports of clinical cases that can be educational, describe a diagnostic or therapeutic dilemma, suggest an association, or present an important adverse reaction.

Other types include methodology articles, debate articles, commentaries, letters to the editor, etc.

Once a PubMed search is conducted, there is also another set of "limits" that may be used. These limits can be viewed and adjusted by clicking on the limits tab just above the search window as shown below:

Below is a screen shot of some of these limits. The most commonly used limit within veterinary medicine is the "veterinary science" limit as show below. This is very helpful to remove articles on human reproduction if desired.

Limits



Google Scholar

http://scholar.google.com/

Google Scholar is a free search engine that indexes scholarly literature across many publishing formats and disciplines. In existence since 2004, Google Scholar indexes most peer-reviewed online journals of Europe and America's largest scholarly publishers.

Unlike PubMed, only keyword searching is possible in Google Scholar. However, Google Scholar will sometimes provide links to the full-text of articles available in institutional repositories, an author's website, or some other publicly accessible site. This is not a possibility with PubMed/MEDLINE.

Advanced search tips are available here: http://scholar.google.com/intl/en/scholar/refinesearch.html The UMN has also created a streaming tutorial on using Google and Google Scholar with a veterinary focus: https://umconnect.umn.edu/p10976343/

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Mechanisms of infection and immunity in the bovine female genital tract post partum

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Abstract

Infection of the female genital tract with bacteria after parturition is common in cattle and in humans. These infections lead to clinical diseases, known as puerperal fever or pelvic inflammatory disease in humans but often called metritis or endometritis in cattle. In cattle, metritis affects 20-40% of animals during the first few weeks after parturition. Clinical endometritis persists in about 20% of animals beyond three weeks postpartum and subclinical endometritis affects many other animals. Clinical disease is characterized by the presence of pus, usually detected in the vagina, and associated with infection of the uterus by *Escherichia coli, Arcanobacterium pyogenes* and anaerobic bacteria. The inflammatory response to infection is dependent on sensing of the microbes by innate immune receptors on endometrial epithelial and stromal cells, which drives an influx of neutrophils to clear the bacteria. However, the innate immune response also perturbs the endocrine function of the endometrium, at least in part by switching prostaglandin secretion from the F to the E series. In addition, cows with uterine disease have abnormal estrous cycles, associated with modulation of ovarian follicle and corpus luteum function. In summary, postpartum bacterial infections are common in dairy cattle and clinical uterine disease disrupts normal fertility by affecting not only endometrial function but also ovarian health.

Keywords: Cattle, uterus, metritis, endometritis, immunity, postpartum.

Introduction

Infection and microbial disease of the female genital tract is common in cattle and humans.[1,2] In humans, postpartum bacterial infections cause puerperal fever or pelvic inflammatory disease (PID), whilst in cattle the terms metritis and endometritis are more commonly used. Sexually transmitted infections are also common where bulls are used in natural mating programs. In humans, sexually transmitted infections are widespread and can lead to PID, whereas postpartum infections, known as puerperal fever, are less common in the developed world. However, puerperal fever has a place in history because the studies of this disease lead to Semmelweis's discovery of antisepsis.[3] In the present review, we lay out our current understanding of infection and immunity in the female genital tract, particularly for the postpartum period.

Comparative aspects of uterine disease

Puerperal fever–infection of the uterus postpartum–was a common cause of maternal mortality for women in the developed world until the dawn of the twentieth century and presently is the cause of 75,000 maternal deaths every year, mostly in low-income countries.[4,5] Ignaz Semmelweis observed that women attended by doctors had fatality rates for puerperal fever of 13 to 18% and this was greater than the 2% mortality for those women cared for by midwives.[3] The high rate of mortality started when junior doctors began performing cadaver dissection, as part of their training, prior to assisting births in the maternity hospital. When the doctors were asked to disinfect their hands in antiseptic solutions before assisting births, the mortality rate fell to about 2%—down to the same level as the midwives. Later Semmelweis initiated the washing of medical instruments and the postpartum maternal mortality rate decreased to about 1%.[3] Despite these clear results Semmelweis was shunned, and it took many years for the role of microbes in disease and the value of antisepsis to become widely accepted concepts. The advent of antimicrobials further reduced the impact of puerperal fever and now it is rare following normal labor in the USA.[4,5]

Puerperal fever is not the only disease associated with microbial infection of the female genital tract in women. Sexually transmitted infections are widespread with ~10% of USA women 16-24 years old having an active sexually transmitted infection and ~340 million new infections across the world each year.[1] Sexually transmitted infections are a common cause of PID, which affects between 1 and 3% of women annually in the developed world.[6-8] A frequent consequence of PID or sexually transmitted infections in humans is obstruction of the Fallopian tubes, which causes infertility.[7] At the time of writing this review, Bob Edwards was awarded the 2010 Nobel Prize in

Physiology or Medicine for developing human *in vitro* fertilization (IVF), and IVF is commonly used to circumvent the infertility associated with obstruction of the Fallopian tubes.[9,10] However, the role of uterine disease in relation to tubal obstruction in cattle is not clear.

Immunity and inflammation not only have a role during infection but also when there is tissue damage. Two important conditions of the human uterus where this applies are menstruation and endometriosis, which are sterile processes associated with inflammation.[11 12] Endometriosis is one of the most common health problems associated with lost days in employment, for example. Comprehensive reviews are available for the mechanisms and consequences of infection, immunity and inflammation in the human female genital tract.[11,12]

Definitions of bovine uterine disease

Pelvic inflammatory disease in cattle is more usually called metritis, although the term for chronic disease–endometritis–is used in humans as well as cattle. The rates of diagnosis of uterine disease continues to increase,[13] whilst common measures of fertility decline.[14] There is an apparent association between increased milk production and reduced conception rates,[14] although the mechanisms and the contribution of uterine disease to this issue are less clear. Of course it is not all bad news for reproductive health in cattle–the development of AI in the second half of the last century all but eliminated sexually transmitted infections in dairy cattle.[15]

Metritis within a week of parturition commonly affects ~20% of animals with maximal herd rates for this clinical disease of 36% and 50%.[16,17] Subsequently, 15% to 20% of cattle have clinical disease that persists beyond three weeks postpartum (endometritis) and about 30% have chronic inflammation of the uterus without clinical signs of uterine disease (subclinical endometritis).[18-21] In cattle, we set out the definitions of disease in 2006 with minor revisions and a grading scheme for metritis in 2009.[2,20] Animals are classified as having grade 1 metritis if they have an abnormally enlarged uterus and a purulent uterine discharge without any systemic signs of ill-health. Animals with additional signs of systemic illness such as decreased milk yield, dullness and fever >39.5°C, are classified as having grade 2 clinical metritis. Animals with signs of toxemia such as inappetance, cold extremities, depression and/or collapse are classified as grade 3 metritis.[2]

Clinical endometritis is defined in cattle as the presence of a purulent discharge detectable in the vagina 21 days or more postpartum, or mucopurulent discharge detectable in the vagina after 26 days postpartum.[20] A simple grading system based on the character of the vaginal mucus is readily used to evaluate cows with clinical endometritis.[20] Vaginal mucus character is graded as 0 (clear or translucent mucus), 1 (mucus containing flecks of white or off-white pus), 2 (exudate containing <50% white or off-white mucopurulent material), or 3 (exudate containing ≥50% purulent material, usually white or yellow but occasionally sanguineous). The endometritis grade correlates with the presence of pathogenic bacteria associated with uterine disease and is prognostic for the likely outcome of treatment.[22 23] However, there is a recent report that up to 38% of cows with pus in the vagina, diagnosed by a Metrichek device, do not have endometrial inflammation as determined by endometrial cytobrush.[24] Further work using a gold standard test such as endometrial biopsy and histology will be needed to resolve the precision of the diagnostic methods.

Subclinical endometritis is defined by polymorphonuclear neutrophils exceeding 5-10% of cells in samples collected by flushing the uterine lumen or by endometrial cytobrush, in the absence of signs of clinical endometritis about five weeks postpartum.[24-27] The incidence of subclinical endometritis is dependent on the diagnostic cut-off used for the proportion of neutrophils in samples and the time postpartum, but is estimated to be on the order of 20 to 50% of animals.[21,24,25] However, there remain many questions about the practicality of diagnosis of subclinical endometritis in the field; the dynamic nature of the diagnosis dependent on time postpartum and other factors such as estrous cycle stage; the mechanisms drawing neutrophils into the uterine lumen; and, how the problem should be treated. Another issue that has emerged from uterine cytology is the agreement between cytology and clinical signs. It appears that if animals are evaluated for ≥ 6% neutrophils in the uterus concurrently with ≥2 score of the vaginal mucus, some animals only have the clinical signs.[24] The implications of this conundrum include the possibility that there are problems with the diagnostic criteria or that pus in the vagina reflects inflammation in the lower genital tract. However, irrespective of these questions, fertility was perturbed by any evidence of inflammation–whether it is ≥6% neutrophils in the uterus and/or ≥2 score of the vaginal mucus.[24] Thus, whilst veterinarians need

to come to a consensus on diagnostic methods, the first step on the critical pathway for developing new treatments for uterine disease is to understand the mechanisms of infection, inflammation and immunity in the female genital tract.

The etiology of clinical uterine disease in dairy cattle

It is assumed that pathogenic bacteria do not reside in the uterus during pregnancy but after parturition the uterine lumen is almost always contaminated with a wide range of bacteria, which are readily cultivable by standard techniques.[28-32] *Escherichia coli* and *Arcanobacterium pyogenes* are the most commonly isolated bacteria, followed by a range of anaerobic bacteria such as *Prevotella* spp., *Fusobacterium necrophorum,* and *F. nucleatum*.[28,31,32] Infection of the uterus with *E. coli* appears to precede infection with other pathogenic bacteria or possibly bovine herpesvirus-4 (BoHV-4).[29,30,33] Furthermore, there are specific strains of *E. coli* that possess a pathogenic potential for causing metritis in cattle, which we term endometrial pathogenic *E. coli* (EnPEC).[34] These bacteria were first characterized in association with disease and their sensitivity to antimicrobials measured.[28,35] We then further identified the bacteria using multi locus sequence typing (MLST) and found they differ from diarrheic and mastitis strains of *E. coli*. The EnPEC strains associated with pelvic inflammatory disease were most adherent for endometrial cells and, like most *E. coli,* expressed functional Type 1 fimbriae (FimH).[34] However, the EnPEC were also invasive for endometrial cells, stimulated a host cell inflammatory response, and could replicate uterine disease *in vivo*.[34] The implications of the discovery of EnPEC provides a paradigm shift for development of vaccines or biological therapeutics for pelvic inflammatory disease, which should specifically target EnPEC rather than other strains of *E. coli*.

Following the EnPEC infection, *A. pyogenes* and anaerobic bacteria cause the most severe endometrial lesions.[36] The impact of the anaerobic bacteria has been harder to quantify but *Prevotella* and *Fusobacterium* species are common in animals with metritis and endometritis. Furthermore, *A. pyogenes, F. necrophorum* and *Prevotella* species appear to act synergistically to enhance the likelihood and severity of uterine disease.[37,38] For example, *F. necrophorum* produces a leukotoxin, *P. melaninogenicus* produces a substance that inhibits phagocytosis, and *A. pyogenes* produces a growth factor for *F. necrophorum*. Unfortunately, antimicrobials that are most effective against anaerobic bacteria are either expensive or not licensed for food production animals.

Bovine herpesvirus 4 is the only virus consistently associated with uterine disease after parturition in cattle.[39,40] Like other herpesviruses, BoHV-4 can establish latent infections in cattle, particularly in macrophages,[41] and the viral infection is often identified concurrent with bacteria that cause uterine disease.[42,43] The virus is highly tropic for endometrial cells, rapidly replicating and killing epithelial or stromal cells.[41,44] The virus may be activated in endometrial cells by cellular pathways stimulated by prostaglandin E_2 (PGE) or by lipopolysaccharide (LPS).[44] Conversely, the virus appears to activate the host gene promoter for interleukin 8 (IL-8) to stimulate the production of this chemokine,[45] perhaps to attract more macrophages that the virus can then persistently infect.

Development of disease is dependent on the balance between host immunity and the pathogenicity of the bacteria. This balance can be tipped in favor of clinical endometritis by risk factors such as retained placenta, dystocia, large calves, twins, and stillbirth.[46,47] Cow-level risk factors identified for subclinical endometritis include ketosis, acute metritis, and the interaction between parity and milk production, in which primiparous cows with higher milk production were at higher risk and multiparous cows with higher production were at lower risk for subclinical disease.[25] The cleanliness of the animal or fecal contamination of the environment appeared to be less important risk factors for endometritis than retained placenta or dystocia in one study.[48] However, in a larger study the herd level risk factors for subclinical endometritis did include environmental factors such as the bedding materials.[25]

The mechanisms linking infection and infertility

Understanding the mechanisms of disease is important for finding new therapeutic or prevention strategies. Furthermore, studying the mechanisms of infection, inflammation and immunity in uterine disease using cattle is biologically relevant because it is a common cause of infertility. Finally, cattle are a useful model to develop concepts that might be applied to humans.[49]

The initial defense of the mammalian endometrium against microbes is dependent on innate immune systems including pattern recognition receptors, complement, antimicrobial peptides, and acute phase proteins.[50] Pattern recognition receptors on mammalian cells bind molecules specific to microbial organisms, often called pathogen associated molecular patterns (PAMPs) or microbial associated molecular patterns (MAMPs).[51-53] The most studied group of receptors is the Toll-like receptors (TLRs), which are most often found in a broad range of immune cells.[53,54] TLR1, TLR2, and TLR6 recognize bacterial lipids such as lipoteichoic acid (LTA) from gram-positive bacteria. TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids often from viruses. Lipopolysaccharide (endotoxin) is the cell wall component of gram-negative bacteria such as *E. coli*, which is bound to LPS-binding protein in plasma and recognized by TLR4 in complex with CD14 and MD-2.[51] TLR5 binds flagellin; and TLR9 also recognizes bacterial DNA. Activation of TLRs initiates the production of pro-inflammatory cytokines and chemokines. The chemokines mobilize and activate immune cells.[54,55] The influx of neutrophils into the uterus is particularly associated with metritis and endometritis.[56] The endometrium from normal non-pregnant cattle expresses TLRs 1 to 10,[57] and the endometrial epithelial and stromal cells express most TLRs.[57] These TLRs appear to be functional as endometrial cells secreted PGE and inflammatory mediators in response to bacterial PAMPs.[58] This LPS-induced PGE secretion by endometrial cells is also important for fertility because prostaglandins have multiple roles in endometrial function, and luteolysis is initiated by prostaglandin $F_2\alpha$ ($PGF_2\alpha$) from oxytocin-stimulated epithelial cells.[59]

The antimicrobial peptides (AMPs) are an ancient component of the immune system and the defensins family are particularly important for mucosal immunity.[60] Bovine uterine tissue expresses lingual antimicrobial peptide (LAP), tracheal antimicrobial peptide (TAP), and β-defensins.[61] Complement is also a likely ancient system with a role in countering uterine infection but little is known about this in cattle. Mucin-1 (MUC1) is an epithelial cell glycosylated transmembrane protein that may also have a role in microbial defense of the endometrium.[62] MUC1 is expressed by epithelial cells of the bovine endometrium and expression was increased when the cells were treated with LPS.[57] Finally, acute phase proteins are produced in the liver in response to circulating cytokines and peripheral plasma concentrations are increased during the first few weeks postpartum in cattle.[63] Acute phase proteins such as LPS binding protein and haptoglobin have roles in immune defense and regulation of immunity.[64-66]

Blood-derived neutrophils and monocytes are the main effecter cells for removing bacteria from the uterus after calving. However, endocrine and metabolic changes around the time of parturition in cattle modulate neutrophil phagocytic function and gene expression.[67,68] Further, blood neutrophils obtained from cows with endometritis are significantly less phagocytic.[69] The process of transmigration into the uterine lumen also modulates neutrophil function; IL8-induced attraction of neutrophils into the uterine lumen increased the generation of reactive oxygen species by these cells.[56] However, when neutrophils are in the uterine lumen their function is further modulated by soluble factors in lochia. Whereas lochia of healthy cows only moderately affected the function of neutrophils, the secretions of infected cows severely depressed the generation of reactive oxygen species.[70]

Changes in hormone concentrations around the time of parturition may influence the risk of uterine infections, and at least the luteal phase or progesterone appear to increases the risk of uterine disease.[71, 72] Dietary energy balance also influences the course of the bovine puerperium, associated with changes in plasma and endometrial levels of insulin-like growth factor 1 (IGF-1).[73,74] Indeed, "modern" dairy cows are selected for milk production, and the increasing capability to produce more milk is associated with decreased fertility.[75] In the last few weeks before parturition and at the start of lactation dairy cows experience decreased dry matter intake (DMI), leading to mobilization of adipose tissue to support milk production. In consequence most lactating dairy cows experience a period of negative energy balance, which interferes with normal reproductive physiology.[76] Decreased DMI and metabolic disturbances around calving are associated with suppression of immune function predisposing lactating cows to bacterial infection.[77] Interestingly, reduced appetite up to two weeks before calving predicted which animals may develop metritis.[78]

Ovarian function is also commonly perturbed in animals with postpartum infection.[28] The oocyte and nurturing granulosa cells grow in a follicle micro-environment where disturbed metabolism can impact fertility. The negative energy balance of lactating dairy cows decreases the

frequency of luteinizing hormone (LH) pulses and is related to low blood concentrations of glucose, insulin and IGF-I. This reduces the production of estradiol by the dominant follicle, impairs oocyte quality and the development of embryos, and limits the production of progesterone by the corpus luteum.[76] Furthermore, cows with uterine infections have slower growth of the first postpartum dominant follicle, lower peripheral plasma estradiol concentrations around the time of maximal follicle diameter, and in those animals that ovulate, peripheral plasma progesterone concentrations are lower after ovulation.[28,29] These effects of uterine microbes on ovarian function could be caused by PAMPs or inflammatory mediators suppressing release of gonadotrophin releasing hormone (GnRH) and the pituitary secretion of LH.[79,80] The follicular fluid of cattle with uterine inflammation also contains LPS and granulosa cells collected from growing or dominant follicles secreted less estradiol when treated with LPS.[81] The effect of uterine disease on follicular function may be further enhanced by cytokines released by the endometrial cells because granulosa cell steroidogenesis is impaired by pro-inflammatory cytokines.[82]

Treatment of uterine disease

A wide variety of therapies for uterine infection have been reported, including antibiotics administered systemically or locally, hormones such as estradiol and $PGF_{2\alpha}$, intrauterine infusions of antiseptics and supportive care including fluid therapy. The perceived success of therapy depends on the elimination of clinical signs and restoration of normal fertility. However, the choice of treatment often causes controversy among veterinarians, perhaps because of the lack of a precise diagnosis, different methods of classifying uterine infections, spontaneous resolution of disease in many animals, and few controlled trials.[19]

Early diagnosis and treatment of animals with metritis is important to control the severity of the disease and limit suffering. Implementation of fresh cow monitoring programs, with daily routine herd health checks to assess appetite, attitude, milk yield and rectal temperature of cows during the first ten days after calving provides an opportunity to identify affected cows. These animals can then receive early supportive therapy in order to maintain DMI during the transition from parturition to lactation. However, solely monitoring the rectal temperature of dairy cows to diagnose metritis is less reliable than including an examination of abnormal uterine discharge. In addition, extra attention needs to be paid to those animals with risk factors for metritis such as retained placenta, dystocia, stillbirths, twins and metabolic conditions such hypocalcaemia and ketosis.[83] Severe, toxic metritis requires urgent and intensive veterinary treatment, whilst mild cases of metritis may require minimal intervention.

Treatment of metritis should aim to eliminate bacteria from the uterine cavity and endometrium without inhibiting uterine defense mechanisms.[84] Metritis is most often treated with broad spectrum antibiotics administered for about three days by the parenteral and/or intra-uterine route, depending on the severity of the symptoms. However, because the tissues deeper than the endometrium are likely affected, the most important line of treatment for metritis is an appropriate parenteral antibiotic that achieves sufficient concentrations in the target tissues. The responsible use of antimicrobial agents in food-producing animals is a concern of many animal health regulatory bodies because of the potential for milk-residue violations, human health risks and development of antimicrobial resistance in bacteria. Bacteria collected from animals with uterine disease are susceptible to cephalosporin compounds.[35] A third generation cephalosporin, ceftiofur, is approved in the USA for systemic administration to lactating dairy cows and the drug reaches all layers of the uterus, the lochia and blood.[84] However, the effectiveness of ceftiofur administration postpartum varies between studies.[85,86] Other cephalosporin compounds and broad-spectrum antibiotics are used for parenteral administration in cows with metritis, such as potentiated amoxicillin. Although oxytetracycline was widely used by veterinarians for the treatment of uterine infections, evidence for bacterial resistance to this antimicrobial and high minimum inhibitory concentrations indicate that oxytetracycline is unlikely to be the optimum treatment.[35] Despite these data, many veterinarians use 2 to 5 g oxytetracycline administered by placing boluses into the uterine lumen for mild cases of metritis, and in one large study this was reported to be beneficial.[87] Finally, non-steroidal anti-inflammatory agents may provide pain relief during metritis but the value of a single injection is limited when fertility outcomes were evaluated.[88]

The rationale for the choice of treatment for clinical endometritis has been widely discussed and remains controversial with many conflicting studies.[23,85,89,90] The commercial reality is that the choice is between no action, intrauterine administration of antimicrobials or antiseptics, and injection of $PGF_2\alpha$ or one of its analogs; the choice is often limited by country and commercial factors. Intrauterine antibiotics such as cephapirin appear to have acceptable clinical cure rates and benefits for subsequent fertility.[23,90,91] Administration of $PGF_2\alpha$ is the treatment used by many veterinarians for cases of endometritis, particularly in animals where a corpus luteum is judged to be present.[23,92-95] However, the efficacy and benefit of prostaglandin treatment for endometritis and the use of prostaglandin during the postpartum period varies considerably between studies.[23,85,90,92-96] The new challenge is to identify the value of treatment of subclinical endometritis and which compounds should be used because the obvious approaches of $PGF_2\alpha$ and/or intrauterine antimicrobials are not particularly rewarding.[97,98]

Summary

Postpartum infections of the female genital tract are an important cause of infertility in modern dairy cattle. The inflammatory response to uterine infection is dependent on sensing of the microbes by innate immune receptors on endometrial cells and the influx of neutrophils to clear the bacteria. However, these responses to the microbes disrupt not only endometrial function but also affect ovarian health. Although there are established treatments available to veterinarians, the emerging data on the mechanisms of disease may provide new insights to prevent disease or limit the impact on reproductive health.

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Theriogenology to enhance animal well-being

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Abstract

Veterinarians practicing the discipline of theriogenology enhance animal well-being in numerous ways. By utilizing reproductive counseling for breeders about genetic selection, and utilizing appropriate techniques to manage breeding, pregnancy and births, reproduction can be optimized such that neonates and their dams have a maximal chance for survival. Additionally, sound breeding management techniques can prevent diseases and injuries that may occur to male or female animals during breeding. Alternatively, veterinarians may utilize techniques to prevent unwanted pregnancies and other conditions that may be associated with reproduction in animals maintained for companionship or work and improve their safety and well-being.

Keywords: Theriogenology, animal well-being, breeding injury, birthing injury

Introduction

Dorland's Medical Dictionary defines health as… "a state of optimal physical, mental, and social wellbeing, and not merely the absence of disease and infirmity".[1] Webster's New World Dictionary defines well-being as well, happy or prosperous.[2] Well-being has been described to involve activities that provide for the physical and psychological needs of animals.[3] The reader is referred to a discussion of animal welfare in relation to feelings, its ability to cope with its environment, stress and health for elaboration of the relationships between animal well-being and animal welfare.[4]

This manuscript provides an overview of procedures and techniques utilized by veterinarians to enhance animal well-being. This paper will review common or potential diseases and injury that animals may incur during various phases of the reproductive process from selection of parents for breeding to the act of breeding, through gestation and parturition and the early postpartum period.

Veterinarians have a myriad of opportunities and skills to reduce or avoid diseases and injuries that may occur during animal breeding. Veterinarians also utilize numerous techniques to confirm pregnancy, to monitor maternal and fetal health during gestation, and procedures to minimize risk for the dam and fetus during parturition and during the early postpartum period.

Male breeding soundness examinations

Breeding soundness examinations are a safe, repeatable systematic method of identifying males that are subfertile or infertile. While the technique of the male breeding soundness examination varies among the species the outcome for utilizing males that are not fertile is the same. Using subfertile or infertile males subjects females to the trauma of repeated breeding and enhances the risk of disease or injury.[5-7]

Injuries in male animals associated with breeding

Numerous injuries may occur to males during breeding.[8,9] Males tend to become aggressive toward other males and protective of females that may be sexually receptive. Stallions, boars, toms and dogs may bite other males and serious fighting may occur, especially in animals that tend to develop pack behavior such as dogs. Additionally, kicking and/or butting are common aggressive acts among stallions, bulls, rams and bucks.[10-11] These behaviors may lead to musculoskeletal or genital injuries that may be life threatening or render the male temporarily or permanently incapable of breeding.

Males may suffer an array of injuries during breeding if there is mismatch between male and female size such that the penis or prepuce may become bruised, develop contusions or minor to severe preputial lacerations. Overly aggressive males or males that are not athletically gifted may suffer rupture of the tunica albuginea of the penis during breeding. Dogs and stallions may additionally suffer severe

genital bite wounds from an unreceptive female during attempted mating. Unreceptive mares may kick the stallion causing minor to severe musculoskeletal injuries or blunt force trauma to the sheath, penis, prepuce or scrotum. Hind limb, pelvic or spinal injuries may occur if the attempted breeding occurs on surfaces that allow slipping or falling during coitus.

Techniques to minimize male injuries associated with breeding

Numerous breeding techniques are utilized to prevent injuries to the male during breeding. Hand mating horses so that both the stallion and the mare are managed by a halter and an assistant may prevent attempted breeding when the mare or stallion is overly aggressive. Mares may be hobbled to prevent them from kicking the stallion. Mares, stallions, and dogs may be muzzled to prevent biting from aggression during breeding.

Maintaining males in single sire units where there is not contact with other males in the breeding area will prevent many injuries. Ensuring environmental protection and sound footing may also alleviate the risk of injury during coitus. Appropriate vaccination prior to breeding can reduce the risk of transmission of some infectious disease from the female to the breeding male.

Diseases and injuries in female animals associated with breeding

Females may suffer numerous musculoskeletal injuries during breeding. These injuries are often related to size disproportion between the female and the male such that females may not be able to adequately support the weight of the larger males during coitus. Resultant injuries may include fractures of the hind limbs, pelvis, spine or soft tissue injuries to joints or nerves. Females may incur genital soft tissue injuries due to size disproportion wherein the penis causes bruising or lacerations to the perineum, vagina or cervix. In severe cases the penis may perforate the cranial vagina with potential fatal results from peritonitis. Less commonly misalignment of the penis with the vulva leads to perforation of the rectum of the female with potentially fatal outcomes. Mares may suffer mild to severe bite wounds from the stallion when he bites her neck or withers during mating.[11]

There are numerous sexually transmitted diseases in animals. Breeding females may contract those diseases either through natural service or in some instances through artificial insemination. A number of heritable diseases that affect different species of animals are due to undesirable genetic defects that affect fetal formation, development and well-being.

Techniques to minimize female injuries associated with breeding

Female breeding soundness examinations allow assessment for normal reproductive tract and skeletal development along with body condition scoring and determination of fitness for breeding. Females that are not considered to be optimal breeders may be withheld from breeding thereby avoiding the risk of diseases or injury due to breeding, gestation or parturition. Part of the breeding soundness examination may include testing for equine infectious anemia or other diseases that may be appropriate for the species.

There are numerous sexually transmitted diseases in animals such as brucellosis and equine contagious metritis and females should be appropriately tested to ensure they are free from those diseases prior to breeding. Breeding sires are also capable of spreading venereal diseases and they should be determined to be free of those diseases prior to breeding or semen collection for artificial insemination.

Females should be appropriately immunized against those infectious agents that pose significant risk of fertilization failure, embryonic or fetal mortality, or fetal infections and for which vaccines are available. The vaccines should be administered sufficiently before breeding to allow an optimal immune response by the female.[12-17]

Pedigree analysis and genetic tests are available for a number of heritable diseases in animals. It is wise to screen females and potential sires for those genetic diseases that affect that particular species and breed of animal. In cattle utilization of genetic selection tools such as Expected Progeny Differences (EPD) allows selection of sires that produce calves of low to moderate birth weights or high calving ease in first calf heifers.[18-20]

One of the most powerful tools to prevent female injury during breeding is the use of artificial insemination. Either using natural estrus or estrus management techniques to schedule ovulation allows females to become pregnant without the physical presence of a male or without coitus. Consequently there is no opportunity for female injury due breeding accidents.

Timing of insemination relative to ovulation may reduce the number of attempted matings to achieve pregnancy. Breeding rolls may prevent excessive penetration of the stallion penis into the vaginal vault of the mare. Additionally shoulder or wither pads or bite pads may prevent injury to mares while being covered by an aggressive stallion. Pharmacological management of the sire may reduce aggressiveness while maintaining libido which may reduce trauma caused by the male during copulation.

Inadequate nutrition during pregnancy is associated with development of a weak or undersized fetus and increased neonatal mortality. Nutritional counseling to ensure that pregnant females are fed to maintain optimal body condition scores during pregnancy may reduce dystocia, prevent pregnancy toxemia, enhance neonatal survival, optimize milk production and hasten return to cyclicity following parturition.

Diseases and injuries in female animals associated with pregnancy and parturition

Metabolic diseases such as hypocalcemia in cows, mares and bitches are associated with advanced gestation and the early postpartum period. A large number of infectious agents are associated with early embryonic death or abortion in different domestic species of animals. Dropsical conditions may occur in pregnant cows, sheep or pigs.

Pregnancy toxemia is usually associated with females pregnant with two or more fetuses in late gestation. This condition may be prevented with early recognition of the presence of twins or triplets, usually with the aid of ultrasound examination, or rectal or ultrasound assessment of an oversized fetus. Vaginal or cervical prolapse in late pregnancy are considered to be heritable conditions in certain breeds of cattle as well as occasionally in ewes and may prevent normal cervical dilation and Stage II labor. The risk of ascending placentitis due to vaginal vault contamination may be minor surgical techniques such as the Caslick's procedure in mares.

Techniques to reduce disease or injuries associated with pregnancy or parturition in female animals

Early pregnancy recognition and termination of an unwanted fetus due to unplanned matings or the presence of twins in mares can eliminate diseases and injuries that the female may experience during pregnancy or parturition. Infections occurring during pregnancy may be detected with physical examinations, routine laboratory testing and perhaps ultrasonography or other imaging modalities. Normal development of the fetus can also be monitored with non-invasive imaging techniques.[24-27]

Appropriate nutrition during pregnancy can generally prevent hypocalcemia and hypomagnesemia which most commonly occur in the last trimester of pregnancy. Pregnancy toxemia due to metabolic energy imbalance is usually associated with the presence of twins or triplets in ewes, does and cows may be avoided by appropriate nutritional support. Additionally, the pregnancy may be terminated early by parturition induction or cesarean section with the goal of preserving fetal viability and the health and well-being of the female. Maternal malnutrition has been associated with the birth of weak puppies and decreased neonatal survival as well as reduced development of abdominal viscera in calves born to undernourished cows.

Females may suffer mild to severe injury during parturition when an overlarge fetus is delivered or the fetus is delivered despite an abnormal posture, presentation or position. Such injuries may include uterine rupture and cervical, vaginal, vulvar or perineal lacerations.

Cervical or vaginal prolapse should be promptly replaced within the vaginal vault and secured such that contamination, contusion, laceration, sepsis, fibrosis or necrosis does not occur that may prevent cervical and vaginal relaxation at the time of parturition. Owners should be offered genetic counseling concerning the potential heritability of these conditions and should be advised to remove affected females and their female offspring in order to reduce the likelihood of the prolapse happening in future generations of their herds or flocks.

Fetal injuries occurring during parturition

Most fetal injuries that occur during parturition are due to relative fetal oversize wherein the fetus is too large to easily pass through the maternal birth canal. Additional injuries may occur when more than one fetus may enter the birth canal simultaneously such as with twins or triplets. Fetal injury may occur due to malpresentation wherein the fetus does not enter the birth canal with the normal presentation, posture, and position. Forceful uterine and abdominal contractions may apply sufficient pressures to cause fetal injury. Weak or absent uterine contractions resulting in prolonged Stage I or Stage II labor may result in fetal stress, hypoxia or acidosis. Excessive traction on the fetus by over-zealous or inexperienced obstetricians may injure the fetus. These injuries range from soft tissue trauma including bruising or nerve injury to fractures of the ribs, vertebrae or limbs.

Increased morbidity and mortality of newborns is associated with inadequate protein and energy intake by the gravid female. Additionally, fetal rumen and omasal development has been shown to be less in calves born to cows that were nutrient restricted during early to mid-gestation than in cows fed appropriate diets. These conditions can be avoided by appropriate maternal nutrition throughout pregnancy.

Techniques to reduce fetal injuries occurring during parturition

Peripartum females should be provided with adequate housing and environmental protection suitable to the species prior to parturition. These females should be monitored frequently to ensure that parturition proceeds in normal fashion. Technologies such as monitoring the calcium content of milk in mares, body temperature in dogs, ultrasound examination for fetal viability or stress or programs such as WhelpWise™ may assist with assessment of fetal and maternal well-being.[28]

Induction of parturition may be initiated to ensure that assistance is immediately available to correct fetal malpresentation, position or posture. Epidural anesthesia may facilitate correction of fetal malpresentation. Episiotomy may be utilized to reduce soft tissue pressure from the vulva or vestibule on the fetus during delivery. Cesarean section may prevent trauma through the birth canal and avoid excessive traction for females with relative fetal oversize, fetal malpresentations not readily correctable per vagina, or for females with pregnancy toxemia, uterine inertia, incomplete cervical dilation, uterine torsion, fibrosis or tumor within the vaginal vault or fibrosis of the vestibule or labia.[29-34]

Through obstetrical intervention veterinarians are often the first to recognize teratogenic or heritable defects that affect fetal viability. Numerous infectious agents cause detectable anomalies in the fetus or alter gestational length. Fescue toxicosis in mares is an example that causes prolonged gestation and dystocia often with edematous fetal membranes that do not rupture in a timely fashion with accompanying fetal asphyxia and agalactia in the mare. Proper nutritional management of the mare along with timely induction of parturition could result in less fetal wastage due to this plant toxin.[35,36]

Summary

The veterinarian is uniquely qualified to provide a holistic approach to reproductive management to enhance animal well-being. This plan includes ensuring that appropriate housing to protect animals from environmental stresses and the availability of appropriate nutrition sources for the animals. Active management of all facets of breeding, pregnancy determination, monitoring of fetal and maternal health during gestation are essential. Provision of an appropriate environment and available assistance at parturition are key components for optimal well-being of the female and her offspring.

Sterilization of male and female animals not destined for breeding can ensure their well-being by altering their behavior to avoid aggression, roaming or territoriality. Sterilization at an appropriate age may also reduce the risk of such diseases as mammary neoplasia, mastitis or pyometra as well as such metabolic diseases as hypocalcemia, hypomagnesemia, pregnancy toxemia.

Finally, by early recognition of abnormal conditions veterinarians may select appropriate testing to confirm the etiology of an abnormality. Veterinarians routinely consult with geneticists, toxicologists,

pathologists, nutritionists and other animal scientists to ensure that owners are appropriately counseled to provide optimal care for the well-being of their animals.

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Cesarean section

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Abstract

The purpose of this article is to remove the surgeon's anxiety and stress associated with performing a cesarean section procedure. In many small animal practices the arrival of a cesarean section heralds total disruption of the daily schedule. Procedural organization into a standard protocol results in a structured flow of tasks that is easily managed throughout the event. The procedure is broken down into several stages with licensed veterinary technicians (LVTs), assistants and doctors trained in a flow pattern that assigns specific duties to key personnel. Thusly assigned, staff members not involved in the procedure can proceed uninterrupted in the daily functions of the clinic. Protocols for emergency and planned cesarean sections will be discussed.

Keywords: Cesarean section, surgery, obstetrics, anesthesia, canine

Stage 1: Scheduling the cesarean section

Emergency cesarean section

No scheduling except a short warning – go to Stage 2.

Elective cesarean section

Using the luteinizing hormone (LH) surge is an excellent method to schedule the cesarean section date. The LH surge occurs when progesterone concentration is approximately 2 ng/ml and marks the initial stage of ovulation in the bitch. Most bitches will whelp at 65 days after the LH surge.[1] Because LH is not measured for all breedings; other methods must be used to determine the date for an elective cesarean section.

We routinely schedule surgery for 59 to 60 days from first breeding date. However, setting the surgery date from day of insemination is not accurate due to the level of maturation of the ova at the time of the breeding. Pregnancy term is 65 days after the LH surge; if the bitch is bred late in the cycle her delivery date is still at 65 days after the LH making pregnancy term several days shorter. When LH is not measured we recommend breeding between days 3 and 6 of estrus, most frequently day 3 and 5 after progesterone reaches 2 ng/ml.[2] When comparing progesterone concentrations with measurement of the LH surge we have found that the LH surge is consistently occurs when progesterone concentrations are 2.0 ± 0.3 ng/ml. If the LH surge is not detected when progesterone reaches 2 ng/ml it may be necessary to utilize another laboratory or method of measurement.

Because serum LH assays are not performed at all breedings, we schedule an ultrasound examination at 23 days following the first breeding date. We confirm pregnancy and we measure the width of the fluid edges of the gestational vesicle at the longest axis of the vesicle. At 23 days we expect the vesicle size to be 1.2 ± 0.2 cm in diameter; the gestational vesicle is still quite round at this stage. At 25 days ultrasound examination reveals gestational vesicles of 2.5 to 2.8 cm diameter giving a noticeable difference in vesicle size between breeding dates. The 25 day gestational vesicle will be oval compared to the 23 day gestational vesicle. Giant breeds may have slightly larger vesicle size at 23 days after a known breeding. Another benefit of the 23 day ultrasound examination is that the gestational vesicles are plainly separated making an accurate puppy count possible. Our puppy counts at 23 days average 80% to 90% accuracy. Fetal death and/or resorption may be found as well as signs of fluid in the uterus that may indicate early signs of pyometra. When scheduling cesarean sections we set the date at 59 to 60 days after breeding[3] to allow for full development of the pups and to avoid the bitch going into labor prior to performing the cesarean section. Labor prior to surgery may increase the stress level of the bitch and cause lowered oxygen saturation of the pups during induction. We may have the owners call daily with resting rectal temperature starting at 55 days after breeding. If the resting rectal temperature drops below

99°F for more than several hours we may schedule surgery directly. Whelp Wise[4] abdominal monitoring is another alternative that may be used to help determine the onset of labor. Because most of the bitches we see have been monitored by progesterone measurement and ultrasound examination early in the pregnancy we have only used Whelp Wise monitoring with bitches that have had a history of dystocia prior to the scheduled cesarean section.

Owners are told to arrive with suitable materials to transfer pups home in any weather. Usually this includes hot water bottles, towels or blankets and a box or cooler that is of sufficient size for the expected litter. We do not recommend using electric heating pads because of the risk for severe burns.

Stage 2: Bitch has arrived at the clinic

The bitch is weighed and given a physical examination. Blood is drawn for a serum chemistry panel. Pre- anesthetic medication is then given. The pre-anesthetic is a mixture of glycopyrrolate and butorphanol.* Glycopyrrolate, an anticholinergic agent, is used because of lowered transplacental passage compared to atropine. Butorphanol is given as initial sedative with pain control to potentiate anesthetic induction allowing lower maintenance levels of inhalation anesthetic. If fetal compromise is suspected, and questions arise about the health of the pups, we will perform an immediate ultrasound examination. A rapid scan is done and heart rates are checked with a Doppler ultrasound to determine if the litter is alive or dead.[5] When heart rates of <170 beats per minute are found we will proceed to surgery as rapidly as possible and will inform the owners that the pup(s) is in fetal distress. If fetal distress is noted the bitch is immediately put on an oxygen mask for 5 minutes. We do not administer the pre-anesthetic medication and go directly to surgery.

If the bitch is anxious she will remain with the owners in the examination room for 5 to 10 minutes allowing time for the pre-anesthetic medication to take effect. The bitch is then transferred to the pre-surgical preparation area. If the owners are inexperienced, we have them wait in the examination room until the bitch anesthetized and on the surgery table. Owners are allowed to watch the surgery from the treatment room through a window into the surgical suite.

Stage 3: Pre-oxygenation and anesthetic induction

The bitch is moved to the surgical preparation area and oxygen therapy is administered using a mask for at least 5 minutes before induction. During pre-oxygenation, an intravenous catheter is placed and the lactated Ringer's solution drip rate is adjusted for the patient's body size, hydration and stress level. Bitches weighing less than 30 pounds are anesthetized with sevoflurane administered by mask because we have found that anesthesia can be induced rapidly and these bitches rarely go through an excitement phase during induction. Fractious bitches or bitches weighing over 30 pounds often have severe and prolonged excitement phases if induced with inhalation anesthetic agents, especially if no pre-anesthetic medication is used. The result of several minutes of excitement and the need for physical restraint may deprive the pups of oxygen until the bitch is intubated and positive ventilation is initiated. The use of ketamine and diazepam for induction is strongly discouraged due to transplacental passage of diazepam. This may cause fetal death or result in extremely challenging neonatal resuscitation. Reversal agents are available for pups that recover poorly, but this raises the question of why should the pups need extra resuscitation efforts, and why have the unnecessary risk of neonatal death due to transplacental sedative agents. The availability of more suitable agents makes the use of ketamine and diazepam unnecessary. Bitches that weigh over 30 pounds are induced with propofol, intubated, and then maintained on sevoflurane. Pulse oximitry and blood pressure are monitored. Once the patient is anesthetized the surgical site is shaved and prepared with an initial scrub; the bitch is then transferred to the surgical suite.

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* Mixture and dose for pre-anesthetic: Butorphenol 10 mg/ml (14ml), glycopyrrolate 0.2 mg/ml (21 ml), given im at 0.5 ml/15 pounds.

Stage 3: Surgery

The surgical field is draped with an initial layer of sterile towels followed by a paper barrier drape. The abdominal incision is routinely made from the umbilicus caudally allowing exposure of the uterus. Initial evaluation of the uterus is done quickly and a pup is exteriorized within a section of the uterine horn. We do not completely exterioirze one or both horns of the uterus before delivery of the first pup. Typically the first uterine incision is placed longitudinally at the middle of one uterine horn allowing for rapid removal of all pups. We routinely remove all pups and all placentas using a single incision in each horn of the uterus. The debate exists as to whether single or multiple incisions are the best for bitch and pups; some surgeons recommend a single incision in the body of the uterus instead of incisions in the horns of the uterus. We have found that a single incision in the uterine body dramatically increases surgical time for large litters and causes difficulty with complete removal of placentas. Leaving placentas during the procedure has not been shown to be detrimental, however, with incisions in both horns, delivery time is dramatically reduced and the removal of all placentas is easily accomplished. If the bitch has not gone into labor before surgery the body of the uterus may be quite small and the chance of surgical damage to the cervix is considerable because the incision needed for puppy removal may include the cervix.

The pup is removed from the uterus and the chorionic and amniotic sacs are opened with fresh gauze. The mouth is cleared and the hard and soft palates are examined for clefts. A gauze pad is placed in the mouth and gentle pressure is placed on the hard and soft palate to clear the nose and throat of mucus. The pup is then examined for any obvious birth defects. A hemostatic clip is applied to the umbilical cord 1 cm from the pup's abdominal wall, a hemostat is placed 1 to 2 cm distal and the umbilical cord is sectioned at about 1 cm from the hemostatic clip. The pup is handed to an assistant on a sterile towel and taken from the surgical suite. The pups are dried with towels and a blow dryer. The nose and mouth are cleared with suction if necessary. The pups are then transferred to a pre-warmed incubator. Swinging of pups is not allowed.[6] When necessary, we use a 25 ga needle in the philtrum to stimulate breathing (Jenchung – GV26). Doxapram is not used due to increased central nervous system oxygen requirements secondary to the medication. All placentas are removed, typically without complication. Traction on the umbilical cord as the only method for placental removal may not be sufficient due to the chorionic attachment. In many bitches the chorionic sac is tightly adherent to the wall of the uterus making removal of the placental tissues difficult. When necessary, we have found that taking the edge of the chorionic sac and gently removing the sac to the level of the placenta will greatly simplify placenta removal. Each pup and its corresponding placenta are removed until the first horn is empty. The second horn is then exteriorized for removal of the remainder of the pups. When the last pup has been removed we give oxytocin (2 to 4 units sq depending on size) and buprenorphine[7] (0.075 mg/10 pounds im). Recent problems with the availability of pain medications may dictate the drug(s) to be used. Because buprenorphine is administered after removal of all pups reversal agents are unnecessary. The uterine incisions are closed using 3-0 or 2-0 poliglecaprone 25 (Monocryl™, Ethicon, Somerville, NJ) in a continuous inverting pattern. Before the uterus is replaced in the abdomen it is critical that a full uterine examination is completed. The uterus is examined both visually and manually for any leakage, both ovaries are visualized and a digital examination of the pelvic area is done to be absolutely certain that all pups have been removed. The uterus is replaced and the muscle fascia is closed with polydioxanone (PDS II™, Ethicon) in a continuous pattern. The subcuticular layer is closed using poliglecaprone 25 in a continuous pattern with deep bites taken to ablate dead space. No skin sutures and no skin glue are used in the procedure. Skin sutures are a potential source for irritation and transdermal contamination.

Stage 4: Recovery and client education

The bitch is moved from the surgery table to a blanket on the floor of the recovery area. After consciousness returns we introduce the pups to the bitch and have all pups nurse under supervision before sending the patient home. During this time a physical examination is performed on the pups and bitch and the pups' palates are checked again and their hearts ausculted.

We have an LVT discuss detailed discharge instructions with the owners. The bitch may be ataxic for several hours and although aggression is rarely associated with current anesthetic agents we still recommend that the bitch be watched closely for the first 12 to 24 hours and not be left alone with her pups until she is completely awake and caring for the pups. All pups must gain weight daily after the first 24 hours. We strongly recommend that all pups be weighed twice daily and the weights recorded for daily evaluation. Pups may lose weight in the first 24 hours but must gain daily starting on day 2. Pups that do not gain weight daily are immediately started on supplemental feeding. We strongly recommend tube feeding and have owners return for a training session. The owners are then told to go home and watch a tube feeding video that we have on our website. We also have a feeding chart that can be downloaded for quick reference on feeding volumes and times.

We have found that dividing the cesarean section into several stages allows for efficient processing of the bitch requiring surgical delivery. The stages also allow for focused training of staff members; this greatly alleviates the stress related to a relatively complicated procedure.

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The successful transcervical insemination

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Endoscopic transcervical insemination (TCI) was pioneered by Marion Wilson about ten years ago.[1] Dr. Wilson recognized TCI as a viable technique and noted that "the learning process will be discouraging". With knowledge of the anatomy and hand/eye coordination the procedure is rapidly and easily performed. Requirements for the procedure are a rigid endoscope with sheath, a light source, and a video camera. The video camera is an integral part of the procedure and any money saved in not buying a camera will be dramatically offset by the difficulty in performing the procedure. The camera allows for rapid and much more radical movement of the endoscope during the procedure.

We utilize a cart with the light source, camera and viewing screen adjacent to the procedure. The use of a 13-inch or larger flat panel television is excellent and will provide a view of the procedure from many feet away. Extremely large dogs are positioned on a rug on the floor and the clinician is seated behind the dog on a low chair. Smaller dogs are on an examination table with a rug and the clinician is standing behind the dog. This positioning allows the clinician and observers full view of the procedure and the video screen.

Transcervical insemination has been shown to be an excellent and rapid insemination technique in the canine.[2] Studies have also been done that show that conception may take place as much as 200 hours after cervical closure.[3] Once mastered, the clinician will have the ability to do the procedure with average time of five minutes or less.

Ovulation is timed in almost all bitches that we breed by serial measurement of progesterone and when frozen semen is to be used we will also measure luteinizing hormone (LH). Pinpointing the ovulatory surge of LH will give a greater degree of accuracy for frozen semen breeding. The bitches will be in standing heat and are receptive to insertion of the TCI endoscope. With the fractious bitch we may use acepromazine or dexmedetomidine in extremely low doses. Very little sedation is required to have a bitch in heat stand without heavy restraint. However, this has only been needed in fewer than ten bitches out of over 2500 breedings.

We use a non-spermicidal jelly on the endoscope and only use enough for ease of insertion. The sheath extends beyond the end of the endoscope allowing for visualization during insertion and insemination. The sheath does not cover the forward or dorsal view of the endoscope because most have a 30^0 angle facing dorsally. A caudal dorsal approach to the vestibule is done for two reasons; the first is to keep the tip of the endoscope away from the clitoral hood that is very painful if pressure is applied, the second reason is to keep the end of the endoscope clear of contamination for better visibility. The endoscope is inserted until it contacts the dorsal aspect of the vestibule and is then rotated upright and inserted over the pelvis in a cranial dorsal direction. When the endoscope has cleared the pelvis the insertion is continued in a cranial ventral motion. The vaginal folds may be examined for crenulation and the insertion is continued cranially. The vaginal vault may be widely dilated or collapsed depending on the bitch. When dilated the insertion to the level of the dorsal median fold is very easy; if collapsed, the endoscope is advanced carefully trying to find the most direct route to the cranial vagina and the dorsal median fold.

As the endoscope approaches the cranial vagina the dorsal median fold will become apparent.[4] The dorsal median fold is a singular fold dorsally in the cranial vagina that is in a cranial caudal linear position and is the landmark for access to the area of the cervix. The cranial vagina will narrow significantly ventral to the dorsal median fold and may cause difficulty with insertion of the endoscope. The smaller diameter endoscopes will have little problem, the larger endoscopes may need rotation and gentle pressure to pass this narrow area to the cervix. The fold is followed and a fissure will be seen dorsally as the start of the cervical tissues. The cranial vagina enlarges again at the cervical os giving room for manipulation of the cervix with the tip of the endoscope. Identification of the cervix is necessary to prevent further advancement of the endoscope to the level of the fornix. The fornix is the

cranial ventral end of the vagina and pain is caused if the endoscope is advanced to this area with even small amounts of pressure.

The cervix is easily identified and may be flat or be significantly pediculated extending into the cranial vagina. The cervix will have a very different appearance from the vaginal tissues and dorsal median fold. The surface will have a rounded "brain coral" appearance with many small folds and, in some cases, what appear as deep fissures around the cervical os. The canine cervical os varies dramatically in position and may appear in any position from straight caudal to straight ventral. Deviation of the cervical os to the right is often caused by a full bladder or colon and may cause inability to catheterize the cervical os. When attempts have failed to catheterize the cervix the endoscope is removed and the bitch is taken outdoors to void urine or feces. Evacuation of the bladder and/or colon will frequently leave the cervical os in a much more accessible position. Insertion of the catheter is also extremely difficult in the bitch with a pediculated cervix.

The tip of the endoscope is used to manipulate the cervix into proper position for catheterization. Wide movement at the operator end may be necessary for proper cervical alignment. We use 8 French 56 cm polypropylene catheters (Sovereign®, Kendall, Mansfield, MA) for almost all inseminations; the cost savings are significant compared to endoscopes needing specialty catheters. In smaller breeds we use a rhinoscope with a 5 French catheter. The cervical os needs to be positioned just dorsal to the viewing area because the catheter is in the channel of the sheath dorsal to the lens of the endoscope. Manual abdominal palpation may also be used to help position the cervix if mobility is a problem.

Sovereign® catheters have a rounded tip with two opposing openings 0.5 cm apart at the end of the catheter. The catheter is inserted in the cervical os to the level of the second opening and the syringe containing the semen is attached. All semen samples to be used are centrifuged (Semen Separating Solution™, Synbiotics, Kansas City, MO) and extended (Fresh Express Extender™, Synbiotics).[5] The total volume is never more than 2 cc and is 1cc to 1.5 cc for smaller bitches. The uterus has a very small lumen and larger volumes of semen may reflux into the cranial vagina or may be lost completely. After the syringe is attached to the catheter the semen column is advanced to the level of the cervix allowing the air in the catheter to escape into the vagina instead of into the uterus. When the semen is at the level of the cervix the catheter is then inserted into the uterus and the semen is slowly introduced into the uterus. During insemination the cervical os is observed for leakage of semen. When leakage is noted decreased pressure is applied to the semen column and the catheter is inserted further into the uterus when possible. When the air column replaces the column of semen at the cervical os it is indicative that all semen is in the uterus. The catheter is left in place and the endoscope is moved caudally into the vaginal vault. The catheter is then removed from the cervix while removing the endoscope from the vagina. Removing the endoscope before the catheter allows the cranial vagina to collapse around the cervix and may help prevent retrograde movement of the semen.

The difficulties encountered during TCI are usually related to the inability to find the cervix or inability to catheterize the cervix when it is visible. Finding the dorsal median fold is crucial to finding the cervix and repeated vaginal examinations will make this a very simple part of the procedure. Once in the vagina the advancement of the endoscope in a cranial ventral direction will assist in progression to the dorsal median fold. In some bitches vaginal and uterine fluids will accumulate in the cranial vagina or in the vestibule at the cervix. This fluid may be removed by suction with a syringe attached to the catheter but in some cases the fluid may need to be flushed out for visibility. We use a 60 cc syringe filled with 0.9% saline and flush the cranial vagina then aspirate the remaining fluid. Visibility is dramatically increased and the saline solution will not harm the extended semen. The catheter may be changed after flushing if needed. We have encountered several dogs in which the vaginal diameter at the dorsal median fold is too narrow for advancement of the endoscope necessitating that the semen be deposited at the cervix. These dogs have not have problems with delivery likely due to relaxin levels at term.

Movement of the cervix with the endoscope is often needed for catheterization. It is important to find the cervical os before starting any manipulation of the cervix. After the endoscope is in contact with the ventral aspect of the cervix it is retracted slightly to relieve pressure on the cervix allowing the tip of the endoscope to be used for manipulation of the cervix in a dorsal direction. In some cases the

manipulation of the cervix will require large range of movement on the camera end. In these cases the camera is critical because of the limited motions allowed when viewing with the eye. If the cervix will not move into position in a relatively short time the endoscope may be retracted to the middle of the dorsal median fold and then turned or aimed to either side to assist in the proper approach to the cervix. With endoscopes that will accommodate the 8 French catheters it is possible to place a 90^0 bend in the catheter about 5 to 8 cm from the tip. The bend is made using heated olive oil in a small container and the tip of the catheter is cut with 0.5 to 0.8 cm of catheter remaining beyond the bend. The olive oil is heated until the catheter will bend but not melt. The catheter will retain the bend after being forced though the inlet on the endoscope allowing the curved end to be rotated while at the cervix. The curve will readily advance into the cervical os. When the catheter is in the cervix a 5 French catheter is placed through the 8 French catheter and advanced into the uterus for insemination.

We have found that certain breeds are very easily catheterized; German Shepherds and Bull Mastiffs may be the best for training while Labrador Retrievers may be among the most difficult. Transcervical insemination is a very simple procedure once the clinician is comfortable finding landmarks and manipulating the cervix. The fear of failure in cervical catheterization may make many clinicians reluctant to do the procedure with owners present. You have invested in the endoscope, camera and all necessary equipment to do TCI work but are worried you cannot catheterize the cervix. Tell your breeders that you will do the TCI for the same fee as a vaginal artificial insemination until you are comfortable with catheterization of the cervix in most of your cases. Have the assistant watch the clock and take only five to ten minutes for the procedure, if the catheter is not in the cervix by that time inseminate at the cervix. You will have provided the client with an excellent vaginal insemination at the least, and with a full TCI at the best, providing yourself with valuable training. Marion Wilson is correct that "the learning process will be discouraging" but repetition will lead to confidence and success.

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Ovarian remnant syndrome – a diagnostic dilemma

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Abstract

Ovarian remnant syndrome is a well-recognized complication of surgical sterilization in dogs and cats. It is characterized by a return of estrus behavior months to years after sterilization. Clinical signs vary widely and may include estrus behavior, vulvar discharge or swelling and attractiveness to males, as well as mammary development and lactation. In some animals, the syndrome is only recognized after the appearance of secondary diseases, such as persistent vaginitis, pyometra and reproductive neoplasia. Differential diagnoses of ovarian remnant syndrome include uterine stump pyometra, exogenous estrogen exposure, vaginitis, urinary tract infections and neoplasia. Suspicion of ovarian remnant syndrome based on clinical signs can be confirmed by several approaches, including vaginal cytologic evaluation, vaginoscopy, hormonal assay, ultrasonographic examination and exploratory laparotomy or laparoscopy.

Keywords: Ovarian remnant syndrome, ovariohysterectomy, GnRH stimulation test

Introduction

Ovarian remnant syndrome is a well-recognized complication of surgical sterilization, accounting for 22-43% of dogs presenting for spay-associated complications.[1,2] Two sources suggest that the syndrome may be more common in cats than dogs,[3,4] however a recent retrospective study did not support this finding.[5] Large breed dogs seem to be over-represented, however no breed predisposition has been documented.[2-5] In one study, ovarian tissue was detected bilaterally in most animals,[3] while several studies report ovarian remnants more commonly in the location of the right pedicle.[1,5,6] Surgical experience has not been documented as a contributing factor to ovarian retention.[5] The most common etiology of ovarian remnant syndrome in companion animals appears to be incomplete removal of one or both ovaries, while less common etiologies include revascularization of small pieces of ovarian tissue at sites distant from the ovarian pedicle and the presence of supernumerary ovaries or the presence of ovarian tissue within the pedicle.[3-5,7-9] Revascularization of ovarian tissue within omentum or other locations is reported more often in cats than dogs.[3,4,10] In a prospective experimental study, eight of nine (88.9%) cats in which ovarian tissue was loosely sutured to the mesentery had functional ovarian tissue six months later.[11]

The most common presenting complaints of animals with ovarian remnant syndrome were related to a return to estrus, including estrus behavior, vulvar discharge or swelling, persistent vaginitis and attractiveness to males.[1,2,4,5,12] Fewer animals presented with reproductive diseases, including pyometra and reproductive neoplasia.[1,2,8,9] The interval from time of surgical sterilization to presentation varied from 17 days to ten years.[3-5]

Diagnostic approach

As with most conditions, the approach to animals presenting for reproductive abnormalities should include a careful history. Most animals with ovarian remnant syndrome have a history of recurrent cyclic clinical signs that are relatively static in severity, whereas occasional animals will have ongoing chronic clinical signs, which may also be consistent with other conditions, such as neoplasia, pyometra or exogenous estrogen exposure. Clinical signs upon presentation vary widely depending on the individual animal, the amount of reproductive tissue remaining in the animal and the stage of the estrous cycle upon presentation. The most common clinical signs observed in reported cases and in our hospital include vulvar swelling, serosanguinous vulvar discharge, estrus behavior (receptiveness to males and flagging in the bitch, vocalization, rolling and lordosis in the queen). Many cases present with more than one clinical sign concurrently,[5] while vulvar discharge is not expected in bitches from which the entire uterus was excised. Additional reported clinical signs include purulent vulvar discharge, mammary development and/or lactation. Many cases present without any active clinical signs, but with historical

clinical signs as described above. Differential diagnoses that should be considered for spayed animals presenting with vulvar discharge or a history of vulvar discharge include ovarian remnant syndrome +/- uterine stump pyometra, exogenous estrogen exposure, vaginitis, urinary tract infections and neoplasia (vaginal, ovarian or adrenal).[4,5,7,13] Diagnosis may be achieved by several approaches, including vaginal cytologic evaluation, vaginoscopy, hormonal assay, ultrasonographic examination and exploratory laparotomy or laparoscopy.

Vaginal endoscopic and cytologic examination

Vaginal examination is particularly valuable in bitches with a clinical presentation consistent with proestrus or estrus. Characteristic changes of the vaginal mucosa under the influence of estrogen include vaginal edema (vaginoscopy), increased cellularity and increased numbers of superficial cells, in combination with low numbers of granulocytes (vaginal cytology), while large numbers of erythrocytes will often be seen in proestrus bitches with a uterine stump. Queens resent vaginal sample collection more than bitches and may require sedation to pass a small swab or perform a small volume lavage. Further, interpretation of vaginal smears from queens may be more difficult than those of bitches, as large sheets of anuclear vaginal cells are usually not seen.[6,14]

At the time of vaginoscopy and vaginal cytologic examination, owners should be carefully questioned about potential sources of exogenous estrogen exposure. Estrogenic creams or medications may be available in the household and it is our experience that regular skin-skin exposure or oral exposure to these medications may lead to characteristic signs of heat and vaginal epithelial changes in spayed animals. Likewise animals may be treated with estrogenic compounds for other conditions, such as urinary incontinence.

Hormonal assay

Hormonal assay is best performed in conjunction with a stimulation test. Assay of a single sample for serum estrogen, progesterone or luteinizing hormone (LH) concentration has not been reliably diagnostic.[4,5,11,15] Elevated serum estrogen concentrations (>20 pg/mL) are consistent with ovarian remnant syndrome, exogenous estrogen exposure and neoplasia.[4,13,14] Conversely, several studies reported cases with behavioral or cytologic signs of estrus which did not have elevated estrogen concentrations.[4,5] Likewise, the absence of elevated serum progesterone concentrations cannot be used to rule out the presence of ovarian tissue. Dogs experience elevated concentrations of serum progesterone (>2ng/mL or >6nmol/L) only during diestrus (for 50-80 days after conclusion of proestrus), whereas cats are induced ovulators and will not have elevated serum progesterone concentrations during interestrus or estrus. Thus, increased serum estrogen or progesterone concentrations are consistent with the presence of ovarian tissue, but low serum concentrations of either hormone cannot be utilized to rule out the presence of ovarian tissue. Luteinizing hormone has been reported to be elevated in animals after ovariectomy or ovariohysterectomy compared to intact animals.[15,16] However, serum LH concentrations rise slowly after ovariectomy and may fluctuate widely during the cycle of animals with ovarian tissue.[16]

In contrast, both gonadotropin releasing hormone (GnRH) and human chorionic gonadotropin (hCG) stimulation tests have been shown to have great diagnostic value in dogs and cats.[4,6,16-18]

Ovulation can be induced reliably with either GnRH or hCG in cats presenting with clinical signs of heat.[4,6,18] England and coworkers administered 500 IU of hCG to ten cats with suspected ovarian remnants and five fully ovariohysterectomized cats and measured serum progesterone concentrations at the time of drug administration and again seven days later. Serum progesterone concentrations were basal in all samples from ovariectomized cats, while they increased from 0.37±0.2 ng/mL to 10.5±9.2 ng/mL in cats with ovarian tissue.[6]

In dogs, ovulation occurs spontaneously and stimulation of ovulation during estrus is not necessary. To confirm presence of functional ovarian tissue and rule out other sources of estrogen exposure after cytologic examination, serum progesterone concentrations may be measured three to six weeks after cessation of clinical signs.

A stimulation test may further be used to diagnose the presence of ovarian tissue in animals which are currently not experiencing clinical signs of heat. Anestrus animals cannot be clinically or hormonally differentiated from neutered animals. However, a sharp increase in estradiol concentration has been demonstrated in dogs and cats that are intact or have retained ovarian tissue after ovariohysterectomy.[17,18] Thus, in the absence of behavioral or cytologic signs to support ovarian presence in either a dog or cat, the combination of a single progesterone assay and a GnRH stimulation test with blood collection before and two hours after GnRH administration would reliably serve to diagnose the presence of ovarian tissue.

Ultrasonographic examination

Ultrasonographic diagnosis of ovarian tissue may be difficult due to the small size of the tissue and variable location. However, ultrasonographic confirmation of ovarian tissue may help guide a surgical approach and can be used to evaluate the uterine stump for evidence of inflammation or infection.[7] In a recent retrospective study by Ball and coworkers, ultrasonography correctly identified the presence (or absence) of ovarian tissue in each pedicle in nine of 12 cases (75%). In three animals, tissue was not detected in one or more locations on ultrasonographic examination, but was subsequently confirmed with histology. In two animals, ovarian tissue was suspected, but subsequently not confirmed at that location.[5] In this study, clinical signs of estrus or proestrus did not appear to affect the ultrasonographers' ability to correctly identify ovarian tissue. However the three dogs that were not in estrus had clinical signs or a history consistent with diestrus. Thus, ovarian tissue may still have been enlarged compared to an anestrus animal, due to the presence of corpora lutea. A granulomatous suture reaction must be considered as a differential for hypoechoic masses in the location of the pedicle and may lead to false positive diagnosis of ovarian tissue.[5]

Exploratory laparotomy

Due to the invasive nature of this procedure, it is recommended that confirmation of ovarian tissue be achieved prior to surgery, however no diagnostic modality described above can reliably diagnose the number and location of tissues in the animal. Thus, even after abdominal ultrasound, a full exploratory examination of the abdominal contents should be performed. Performing the laparotomy during behavioral estrus or during diestrus may enhance the surgeon's ability to grossly see ovarian tissue in the pedicles or omentum. However, if ovarian tissue is not detected, excisional biopsies of the pedicles should be performed. Ovarian and other tissues removed during the exploratory laparotomy should be submitted for histopathologic examination. Several cases have documented neoplastic changes in retained ovarian tissue, as well as other reproductive organs of animals with ovarian remnant syndrome.[5,8-10,14,19] Ball and coworkers further reported increased incidence of neoplastic changes in ovarian remnants (23.8%) compared to sexually intact female dogs (6.25%).[5]

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Pregnancy termination in companion animals

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Abstract

Despite a large body of research and numerous review papers on the subject, there is still a great disparity in treatment protocols for mismated companion animals in the USA and elsewhere. Numerous treatment protocols are available. Ovariohysterectomy has the advantage of permanently removing the risk of an unwanted pregnancy. Medical treatments have varying side effects, depending on the protocol selected, and depend on owner vigilance to prevent future pregnancies. Medical treatment is most often performed in early gestation or mid-gestation. Pregnancy termination after fetal ossification results in abortion of non-viable or poorly viable fetuses.

Early treatment, prior to or shortly after onset of diestrus, results in resorption of fetal fluids and tissues without vulvar discharge, but must be done prior to pregnancy diagnosis. Treatment protocols for pregnancy termination at this stage are associated with more side effects than those for mid-gestation. Pregnancy termination after ultrasonographic confirmation in mid-gestation may result in resorption of all fetal tissues or in expulsion of bloody discharge. Delaying medical treatment until after pregnancy diagnosis minimizes drug risks by avoiding treatment of non-pregnant animals and through the use of lower doses of medication. Several protocols for pregnancy termination at this stage are discussed in detail in this review article.

Keywords: Pregnancy termination, prostaglandin, dopamine agonist, estrogen, glucocorticoid, GnRH

Introduction

Veterinarians in all walks of life–from those that focus on companion animal reproduction to large animal veterinarians with no other small animal experience–are regularly asked "*what to do?*" with a bitch or queen that escaped at just the wrong time. Furthermore, in a time where thousands of unwanted animals are euthanized daily in the USA, finding the answer to that question carries a real significance and has stimulated a wealth of research and numerous reviews.[1-9] However, little consensus exists regarding the best approach to pregnancy termination in dogs and cats.

Determining the risk of pregnancy

Treatment choices should be guided by an animal's actual risk of pregnancy. In dogs, a vaginal swab and cytologic evaluation provide two key pieces of information: 1) detection of sperm heads on cytology can confirm exposure to a male, and 2) determination of estrus stage at the time or presentation. In a study involving 16 females with known breeding histories, Whitacre and coworkers demonstrated that sperm could be seen microscopically in 100% of cases within 24 hours of breeding and in 75% of cases within 48 hours of breeding, using a modified sampling technique.[10] In this study, a moistened swab was placed into the vagina for 60 seconds before removal and then submerged in 0.5 ml of saline for ten minutes. The saline was centrifuged and sediment was examined after staining. Determination of estrus stage is also a key component of determining the risk of pregnancy. Bitches with known or suspected exposure to a male during proestrus are at much lower risk of pregnancy than those exposed during estrus. Cytologic diagnosis of proestrus can be further confirmed with serum progesterone concentrations of <2 ng/mL. In cats, collection of a sample and interpretation of a vaginal cytology is more difficult than dogs and it is not known whether sperm can be easily detected in this species. However, as cats are induced ovulators, serum progesterone will likely be low in unexposed cats (<2 ng/mL) and would rise after coitus in exposed cats.

During the examination, owners should be questioned regarding the intended use of their animal and the relative merits of different treatment options should be explained. Animals that are not intended to be breeding animals should undergo ovariohysterectomy in early diestrus or pregnancy. This will prevent future unwanted pregnancies and may decrease the animals' risk of several diseases, including pyometra and mammary and ovarian neoplasia.[7]

Early pregnancy termination

Several protocols have been proposed to medically treat bitches as soon as a mismating occurs.[11-14] This approach has the advantage of rapidly addressing the owners' concerns and preventing pregnancy, but may pose a greater risk to the animal and have lower success rates than other protocols. As a result, most reviewers have recommended against these treatment regimens.[3,6,7,9,15]

Estrogens have been widely used for this purpose in the past, but potentially severe side-effects should raise concerns. In a study by Bowen and coworkers, which demonstrated a high efficacy of this drug, two of eight dogs receiving estradiol cypionate developed pyometra.[11] More recently, Whitehead reported that the risk of pyometra in animals treated with low doses of estradiol benzoate was more than six times greater than the risk in untreated animals.[16] Further, several reports indicate significant other health risks associated with estrogen treatment in dogs and cats, including infertility and severe myelotoxicity.[11,17,18]

Prostaglandins are effective at inducing luteolysis early in diestrus.[4,13,19,20] However, the high doses of prostaglandin required in early pregnancy are associated with substantial clinical side effects, including emesis, diarrhea and cramping. Further, pregnancy may be maintained even after documented luteolysis at this stage.[4,20,21]

In contrast, work in recent years shows good success for other treatment regimens in early pregnancy. These are currently not available in the USA, but may pose viable treatment options in the future. The progesterone antagonist aglepristone is licensed for veterinary use in Europe, but is restricted in the USA. Antiprogestins have been shown to effectively induce fetal resorption with minimal side effects in early and mid-gestation and fetal abortion in late gestation.[1,6,14,22-25] Recently, a series of studies by Gobello and coworkers have investigated the effects of the gonadotropin releasing hormone (GnRH) antagonist acyline in dogs and cats. Although this drug is currently not commercially available, several studies suggest that it may be useful to prevent pregnancy if given to either a bitch or queen during proestrus with few side effects.[26,27]

Pregnancy termination in mid-gestation

Due to a lack of (known) early pregnancy factors in companion animals, which would aid pregnancy diagnosis, any treatment protocol at this stage must be done based on the suspicion of pregnancy. However, in one retrospective study, fewer than 40% of animals that presented because of mismating were pregnant.[21] Thus, it is likely that even within the pool of high risk animals (based on cytologic and hormonal diagnosis of sperm and estrus), a proportion of animals would be treated needlessly. This can be avoided by delaying treatment until pregnancy diagnosis can be achieved.[7] In the dog, pregnancy can be readily diagnosed by palpation approximately 30 days after mating, whereas ultrasonographic examination can confirm pregnancy as early as 15-18 days after the luteinizing hormone (LH) surge (~10-20 days after mating). Embryos are easily detectable between day 22 and 25 after LH surge.[28,29] In the queen, ultrasonographic pregnancy diagnosis can be achieved as soon as 11 days after mating and the embryo becomes visible by 14 or 15 days after mating.

Five major drug classifications have been described for pregnancy termination in mid-pregnancy: prostaglandins (natural and synthetic), dopamine agonists, antiprogestins, glucocorticoids and GnRH antagonists. At this stage, embryonic fluids and tissues are resorbed by the uterus and few clinical signs are expected in response to medical intervention. A bloody discharge may be seen in bitches and queens after approximately 30 days. Abortion (fetal expulsion) occurs after 40-45 days, when fetal ossification is underway.[8,28]

Prostaglandins

In domestic carnivores, pregnancy maintenance is dependent on luteal progesterone production. Although small amounts of progesterone are also produced by the feline placenta, this does not appear to be sufficient for pregnancy maintenance.[30]

Natural prostaglandin can be used to terminate pregnancy beginning five days after ovulation, however prior to 25 days, higher doses are required than later in gestation. Common side effects include hyper-salivation, bradycardia, emesis and reflex voiding and are more severe in dogs than in cats.[4]

Hospitalization and careful monitoring of animals are recommended to control side effects, which tend to be more severe early and diminish during the course of treatment. Side effects may be minimized by using low doses (30-50 μg/kg, 3-4x daily for 4-11 days),[4,13,31] or by starting with a lower dose and gradually increasing it (30-50 μg/kg, increased to 100-200 μg/kg).[7] It should be noted that low doses may not induce permanent luteolysis, resulting in loss of only some pups or fetal death followed by mummification.[4] The synthetic prostaglandin cloprostenol has also resulted in effective pregnancy termination, with few side effects.[6,9,31] In the USA, most practitioners are familiar with protocols using 100-250 μg/kg of prostaglandin 2-3x daily for 4-6 days.[4,20,21]

Dopamine agonists

In both the bitch and queen, prolactin plays a necessary luteotrophic role in pregnancy maintenance.[6,15,32] Progesterone can be reduced or eliminated during late pregnancy by administering a dopamine agonist, which inhibits endogenous prolactin secretion.[32] However, treatment success for pregnancy termination has been inconsistent in both dogs and cats and dopamine agonists alone are rarely used in companion animals.[9]

Progesterone antagonists

Progesterone antagonists including RU 486 (mifepristone) and aglepristone have been studied extensively and result in pregnancy termination prior to 45 days with minimal side effects in dogs.[2,14,22,23,25] Aglepristone also was effective for pregnancy termination in the cat, with higher success rates early in pregnancy than late in pregnancy.[1] Aglepristone is licensed for veterinary use in Europe, however progesterone antagonists are currently not available for veterinary use in the USA.

Glucocorticoids

Administration of oral and injectable formulations of dexamethasone for ten days to pregnant bitches between 28 and 51 days of gestation resulted in resorption or abortion in all treated animals.[33,34] Subsequent studies involving twice daily administration of dexamethasone (200 μg/kg, tapering over the last 3 days) for 7.5 or 9.5 days resulted in pregnancy termination in up to 100% of cases.[35] Premature and term delivery of live or dead fetuses resulted in some cases of animals treated after 45 days, while vaginal discharge was the primary clinical sign in most dogs treated in mid-gestation. Polyuria and polydipsia were the primary side effects noted in these studies and attributed to adrenal suppression. Side effects were transient and disappeared after conclusion of the treatment. Glucocorticoids have the advantage of being inexpensive and are often available in oral formulation. Thus they are easily administered to animals on an outpatient basis and can be administered in food to feral animals. The mechanism of action has not been fully elucidated at this time. The use of glucocorticoids for pregnancy termination has not been investigated in cats.

GnRH antagonists

The concept of using GnRH agonists or antagonists for the purpose of pregnancy termination was first described by Vickery three decades ago.[36] At that time, hypersensitivity reactions in dogs prevented their use. Recently, however Gobello and coworkers have shown that a third-generation GnRH antagonist, acyline can terminate pregnancy in dogs.[37,38] Twenty-one animals were administered 110 μg/kg or 330 μg/kg of acyline, or saline via a single subcutaneous injection between 25 and 35 days of gestation. All animals that received acyline, but no animals injected with saline experienced an abortion between two and 12 days after treatment. This approach would potentially be highly valuable for feral populations of dogs, where repeated administration of medication is difficult, but is currently not commercially available. It is not known at this time, whether earlier administration of a GnRH antagonist during pregnancy such as acyline would result in resorption rather than abortion. Acyline was not effective at inducing luteolysis or pregnancy termination in queens.[27]

Combined prostaglandin/dopamine agonist regimen

Extensive work by Verstegen and coworkers, demonstrated that a dual approach to pregnancy termination results in reliable efficacy in mid-gestation.[19,24,39] Low doses of either natural or synthetic prostaglandin results in luteolysis, while dopamine agonists inhibit prolactin release. In a series of studies, administration of cabergoline (1.7-5μg/kg once daily for 10 days) and cloprostenol (1-2.5 μg/kg once or twice 5 days apart) resulted in fetal resorption with minimal discharge or unwanted side effects.[19,39-42] In cats, pregnancy was terminated in five of five animals administered cabergoline (5 μg/kg once daily) and cloprostenol (5 μg.kg every other day) to effect.[24] In both dogs and cats, treatment was continued until ultrasonographic confirmation of fetal demise. The above protocol has several distinct advantages over other protocols described. Both prostaglandin and dopamine agonists are readily available in the USA. The combined luteolytic and antiluteotrophic mechanisms decrease dosages of each drug, substantially reducing side effects. The protocols can be instituted around day 25 days and result in resorption prior to fetal ossification, when most animals would abort formed fetuses. The use of orally administered dopamine agonists and long-acting, synthetic prostaglandin eliminates the need for frequent examination and hospitalization.

Conclusions

In conclusion, risk for pregnancy should be determined at the time of mismating and ideally pregnancy should be confirmed prior to treatment in order to avoid unnecessary and potentially harmful medical side effects. Treatment choices should be based on the animal's stage of gestation and drug availability and should be tailored to minimize side effects as much as possible. A treatment onset between 25 and 30 days and combination of two drugs, including a prostaglandin and dopamine agonist minimize drug dosage and side effects while inducing fetal resorption rather than abortion. Alternate treatment protocols, including the use of progesterone antagonists of GnRH antagonists may become available in the USA in the future.

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Infertility and subfertility in the male dog

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Introduction

Infertility and subfertility in the male dog is one of the most frustrating problems dealt with by the theriogenologist. Semen quality is difficult, if not impossible, to influence. When problems are suspected, the clinician must do a complete examination to determine the fertility status of the dog, as well as the potential cause(s) of the problems detected. Some causes of male dog infertility may be reversible, where most are not. If a bitch does not become pregnant after breeding, the dog must be considered as a potential reason for that infertility. Semen quality and breeding management account for most unsuccessful matings in the bitch. As part of the diagnostics applied to canine infertility, a breeding soundness examination of the dog must be performed.[1]

Keywords: Breeding soundness, infertility, semen evaluation, canine

Breeding soundness examination[1-3]

History

Breeding soundness examinations of the male dog should begin with a complete history of that individual dog. Health and breeding history may be helpful in identifying the cause and chronology of any problems. General health issues can influence the function of the reproductive tract of any male. Systemic disease, including any that might cause fevers, can adversely affect the functioning of the testes. Endocrine disease can disrupt the feedback mechanisms responsible for the production of sperm and hormones in the testes.

Physical examination

A physical examination of the dog is performed including the internal and external genitalia. The scrotal contents include the testes, epididymes, vaginal tunics, and the spermatic cord. The spermatic cord contains the pampiniform plexus surrounding the testicular artery, vas deferens and the cremaster muscle. The thermoregulatory properties of these structures keep the testes at a temperature that is less than body temperature combined with the ability of the tunica dartos of the scrotum itself to contract and relax. This reduced temperature is required for proper functioning of the testes in making spermatozoa. Testosterone is typically unaffected by the temperature of the testes and will continue to be in the normal range even in the event of abdominal testes. The only internal structure of importance is the prostate gland. The prostate gland is the only accessory sex gland in the dog and is often the site of problems. The penis and prepuce is examined for normal anatomy, injuries, and evidence of inflammation.

Manual ejaculation

Most intact male dogs can be manually stimulated to ejaculate. There are three fractions to the dog ejaculate, the presperm, sperm-rich, and postsperm fractions. The presperm and postsperm fractions originate from the prostate gland. The sperm-rich fraction comes from the testes through the epididymes and the vas deferens. Evaluation of the ejaculate starts with noting the color of the ejaculate. The normal ejaculate will be grayish to white in color, depending on the concentration of sperm cells. Any other color indicates contamination of the ejaculate with such fluids as urine (yellow), blood (red or brown), or pus. The presperm fraction of the ejaculate is unpredictable in volume, depending on the level of sexual excitement of the dog or the health of the prostate gland. Typically, the presperm fraction is collected until followed by the sperm-rich fraction, collecting the first and second fraction together. The postsperm fraction is normally crystal clear and follows a pause in the pulsations. Any deviation from a water-like appearance in the prostatic fluid indicates problems.

Semen assessment

Sperm quality in the dog is evaluated by assessing motility and morphology of the spermatozoa. Motility is assessed on a warm slide with 10-40X objectives of bright field or phase-contrast microscopes. An attempt is made to estimate the total percentage of motile cells expressed by a percentage. Morphology is assessed with the 100X (oil immersion) objective after staining a drop of semen. Oil immersion (1000X) magnification is essential to correctly assess sperm morphology. The stain used is critical. Eosin-nigrosin (Hancock) stain is an excellent stain for semen. It is easy and inexpensive to use and readily available. Many veterinarians feel comfortable assessing motility, but neglect the assessment of sperm morphology. Major problems are missed when sperm morphology is not part of the semen evaluation therefore must be part of the semen assessment of the dog. Sperm quantity is measured by volume and concentration. Volume can be measured by aspirating into a syringe or pipetting the ejaculate into a graduated tube. Concentration is measured by the use of a hemocytometer, the gold standard.[4] The number of total motile normal sperm cells in the ejaculate can be calculated by multiplying these four measurements together: Volume X Concentration X Percent Motile X Percent Normal. An estimated adequate breeding dose in the dog is 200 million normal motile cells. This number is probably reduced in small breeds due to the small size of both the dog and bitch.

If the semen output is found to be lacking in quantity or quality, then the fertility of that male dog is diminished. A low number of sperm cells is termed oligospermia. The presence of abnormal sperm cells is termed teratospermia. Poor motility, asthenospermia, can also affect the fertility of a dog. Lack of sperm in an ejaculate is azoospermia, the only condition that can be considered truly sterile.

Semen handling

When handling semen, care must be taken not to damage the sample. The major categories of iatrogenic damage to sperm cells are temperature, chemical, mechanical, and osmotic. Semen samples in the dog can be maintained at room temperature for several hours without significant damage to the sample. Extremes in temperatures are usually not a problem when handling dog semen, except in the case of overheating of equipment. Chemical damage of sperm is to be avoided. Reusable equipment is typically avoided due to the chance of detergent residues left after washing. Another source of chemical damage is with the use of lubricants that contain bacteriostatic compounds, (e.g. chlorhexadine). Osmotic damage is seen whenever semen is exposed to water. Care should be taken whenever water baths are used not to contaminate the semen with water. Mechanical damage can occur when aspiration or expression of the semen is performed too quickly. Slow aspiration of semen into or out of a syringe should be a goal.

Causes of infertility/subfertility

Once a dog has been evaluated and found to have problems with sperm quality or quantity, an attempt is made to determine the cause. Many times, a cause cannot be identified. The spermatogenic cycle in the dog is 54 days.[5] The ejaculate obtained on a particular day has been forming and developing for the last two months. Any condition or insult to the dog that has occurred during that two months will influence his semen quantity and quality. In order to assess prognosis, sequential samples may be taken at two-month intervals. The progression of a problem or of recovery can only be determined by serial evaluations.

Testicular degeneration

Testicular degeneration is a general term given to testes that have undergone some kind of decline from normal. Palpation reveals a softening of the testes and a lack of tone. Over time, testicular degeneration can result in a decrease in size of the testes, ultimately resulting in testicular atrophy. There are a multitude of causes of testicular generation. The most common one found in the dog is age-related testicular degeneration. As the dog ages, the semen quality will eventually decline. Any dog over the age of five is at risk for age-related testicular degeneration. One common morphologic abnormality seen in

the semen of older dogs is proximal droplets. Some older dogs will have an ejaculate with 100% proximal droplets leading to very reduced fertility, if not sterility. Often, these ejaculates have very good motility that will lead many evaluators to misjudge the fertility of the dog. Other individuals will show a progressive percentage of a variety of sperm abnormalities in the ejaculate. Age-related testicular degeneration is almost always irreversible.

Reversible testicular degeneration is seen when there is a correctable problem with the thermoregulation of the testes. This can be caused by high environmental temperature, ex. summer months, or by rigorous training. Show dogs are often exposed to hair dryers that can cause overheating of the testes. Systemic diseases that cause fevers or any inflammation of the testes or the scrotum can cause testicular degeneration. Skin disease can affect the scrotum's ability to cool the testes. If a problem in thermoregulation is identified and corrected, the testes may recover and produce a normal ejaculate. As stated above, prognosis can only be accurately determined by serial evaluations.

Prostate gland disease

Intact male dogs produce testosterone that, in turn, causes an enlargement of the prostate gland. Being an accessory sex gland, the prostate gland is responsive to and stimulated by testosterone. The prostate gland, in turn, provides the volume of the canine ejaculate. Benign prostatic hypertrophy/hyperplasia (BPH) is a common finding in male dogs and may be a normal finding in many individuals. Attempts to determine the normal limits of prostatic size in intact male dogs have been made and debated. Due to the extreme body size differences seen among breeds, prostate gland size is variable and dependent on the size of the dog. Benign prostate enlargement typically does not cause any problems for the dog or his fertility. Benign prostatic enlargement will sometimes lead to the development of intraprostatic cysts by occluding ducts leading to the prostatic urethra.[4] At this time, prostatic enlargement cannot be referred to as benign. Prostatic cysts may become contaminated with bacteria leading to prostatic abscesses. Abscesses of the prostate gland are life threatening and difficult to treat.

Another common finding in intact male dogs is prostatic bleeding. Prostatic bleeding is often brought on by exposure to cycling bitches and the sexual excitement generated by this exposure. Blood is seen in the ejaculate and in voided urine. Prostatic bleeding does not necessarily come from dogs with BPH. On rectal palpation, these prostate glands are often normal in size and nonpainful.

Ultrasonography is a useful diagnostic tool for evaluating the prostate gland. Prostatic cysts, abscesses, or neoplasia can easily be identified by ultrasound examination. Ultrasound guided aspiration of the cysts transabdominally can help make the diagnosis of bacterial infection. Urinanalysis by cystocentesis is also helpful in identifying any bacterial component to the disease process as bacterial infections tend to occur simultaneously in the urinary bladder and the prostate gland. Bacterial infections should be confirmed prior to the use of antimicrobials.

Castration was once the only treatment for prostatic disease in the dog. With the discovery of 5 alpha reductase (5-aR) inhibitors, prostatic enlargement and prostatic bleeding are easily treated in those dogs that are still usable and desirable as breeding stock. Testosterone is converted to dihydrotestosterone by 5-aR within the prostate gland. Dihydrotestosterone is the hormone that most affects the prostate gland. By inhibiting 5-aR, the effect of testosterone on the prostate gland is effectively blocked.[6] Finasteride (5 mg/day) and megestrol acetate (0.25 mg/lb/day) are two commonly used drugs which will inhibit 5-aR. Given orally for six to eight weeks, both compounds will dramatically shrink the size of the prostate gland. It has been the experience of the author that megestrol acetate is more effective than finasteride in clearing blood from the ejaculate. Megestrol acetate has more effect on libido than finasteride. If the dog needs to be mated during treatment, finasteride might be a better choice. Treatment with either drug can be repeated as needed over the course of the dog's life.

Azoospermia

Azoospermia is the lack of sperm in an ejaculate. In order to confirm this diagnosis, several tests should be run. Urinanalysis (U/A) will confirm the presence or absence of sperm within the urinary tract. Intact male dogs almost always have sperm in their urine. In addition to the U/A, alkaline phosphate

(ALK) can be measured from the seminal plasma. Alkaline phosphate originates from the epididymes and should be high (>1000) if the second fraction of the ejaculate has been collected. If the second fraction has been collected yet there are no spermatozoa present, a diagnosis of azoospermia can be made. If ALK is low (<100), an incomplete ejaculate has been obtained and a repeat ejaculation is needed. Some dogs are timid or resentful of manual ejaculation. Dinoprost (prostaglandin $F_2\alpha$), can be used in those individuals that refuse to respond to manual stimulation with an ejaculation.[7] If a diagnosis of azoospermia is confirmed, the dog is sterile. Azoospermia is rarely reversible. Often, azoospermia is congenital.

Oligospermia

Low numbers of sperm in the ejaculate of the dog is a finding confirmed when sperm concentration is measured. Sperm numbers in males is a factor of testicular size and mass. More testicular tissue gives the ability to produce greater numbers of sperm.[9] If dogs give $<200 \times 10^6$ sperm cells, ($<50 \times 10^6$ in toy breeds), the diagnosis of oligospermia is made. Some males will experience retrograde ejaculation, putting numbers of sperm into the urinary bladder.[8] Diagnosis of retrograde ejaculation can be made by examining the urine after manual collection and determining the number of sperm contained within the urinary bladder. Treatment of retrograde ejaculation can be accomplished by the use of a sympathomimetic agent, (i.e., pseudoephedrine hydrochloride 4 mg/kg PO) one and three hours prior to semen collection.[10]

If oligozoospermia is diagnosed, and no other cause is found, the author has been able to treat oligospermia with injections of gonadotropin releasing hormone over a four to six month period (i.e. gonadorelin 3 μg/kg im once weekly). The author has been able to double or triple sperm output in some individuals with this treatment.

Bacterial infections

Bacterial infections within the male genital tract can occur. Most infections will involve the prostate gland along with the urinary tract. Some infections will ascend up the vas deferens into the epididymes and testes while others will infect the reproductive tract by the hematogenous route.

Brucella canis

Brucella canis is the number one rule out for orchitis and epididymitis in the dog. Brucella can be spread by venereal or by oral contact with the organism. Brucella in males prefers the testes and epididymes most likely due to erythritol present in these tissues. Erythritol is a preferred nutrient for the Brucella organism. Brucella is an intracellular organism, thus very difficult to clear from the body. Since the reproductive tract is the primary target, Brucella quickly causes damage to the fertility of the male dog. Acute infections are evident by orchitis and epididymitis, while chronic infections lead to testicular atrophy. Breeding stock is lost permanently to *Brucella canis*. Euthanasia is considered the treatment of choice due to the infectious nature of the disease to other canids and its zoonotic potential. Long-term antimicrobial therapy with castration can be a second treatment choice if care is taken to prevent contact with susceptible populations. Yearly serology is suggested if this route is taken to identify any recrudescence of the organism.

Miscellaneous bacterial infections

Any orchitis can cause infertility that may be permanent. Bacterial infections of the male dog's reproductive tract include organisms that are documented as normal flora. Mycobacterium has been shown to be normal flora in the dog and bitch's reproductive tract.[12-14] In certain situations (i.e. high population density), mycobacteria can cause problems in semen quality. Husbandry is a primary mode of management, by reducing the population density. Only then can antimicrobials help in eliminating the problem. Without attention to husbandry, antimicrobials are only a stop-gap treatment, with recrudescence expected due to environmental contamination of the organism. Blastomycosis has been shown to cause orchitis in affected dogs, but only in 2% of the cases.[11] Blastomycosis has been known to

affect the testes and epididymis. It is a complicated disease which is difficult and expensive to treat. Most cases of blastomycotic infections result in loss of functional testes. Castration may be required as part of the treatment in any case of orchitis. Unilateral castration can be considered if the problem is affecting only one testes. A single testes has been shown to produce 75% of the sperm that two can produce due to hypertrophy of the remaining testes after surgery[15]. Orchitis caused by penetrating injury is treated like any other infection. Prognosis for fertility is guarded until treatment is complete and the damage to the testes is known.

Conclusions and potential treatment

Treatment in a subfertile or infertile male is often futile. Infertility and subfertility have a variety of causes in the male dog. Unless a cause can be identified and corrected, reversal is unlikely. Only time and sequential examinations will give an accurate prognosis. Age-related testicular degeneration is the most common cause of infertility in the dog seen in the author's practice and is rarely reversible. Problems related to thermoregulation are also common (i.e. seasonal infertility related to hot summer temperatures). It is not unusual for a dog to be subfertile in the fall months and fertile in the spring months due to summer heat stress. If poor quality semen is seen in the late summer, additional evaluations are indicated after several months of cooler weather to document this seasonal effect. Another potential treatment for testicular degeneration is acupuncture. Acupuncture has been shown to positively affect the reproductive tract in humans and horses.[16-18] The author has seen remarkable results in reproductive cases that normally would have had no hope of resolution. At this time, no one definitively knows how acupuncture affects the reproductive system. It is theorized that acupuncture causes a normalization of the autonomic nervous system.

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The physiology of ovulation timing in the bitch

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Abstract

Accurately timing breeding of a bitch is often done using vaginal cytology, vaginoscopy, and serum progesterone assays, none of which is a direct measure of ovulation or oocyte fertility. Despite the lack of direct connection to fertility, the indirect connections are strong and predictable and have to do with the effects of estrogen and progesterone on the bitch's anatomy and physiology. This review discusses the effects of estrogen and progesterone on the bitch and how the changes we use to predict when to breed are related to the fluctuations in these hormones.

Keywords: Breeding management, vaginal cytology, vaginoscopy, fertility, estrogen, progesterone

Introduction

Breeding management is probably the most common reason for evaluating a bitch in the context of reproduction and fertility. This is appropriate, as many bitches presenting for perceived subfertility have merely not had adequate monitoring and management of their previous estrous cycles.[1,2] Conducting a thorough breeding management on the next estrous cycle of these bitches will often result in pregnancy. The variable lengths of both proestrus and estrus in the bitch account for the high number of miscalculated breeding dates (five–20 days and six–11 days, respectively).[3] The actual fertile period, when oocytes are at a stage where they are fertile and accessible by vaginal deposition of semen (as is the case in natural mating and traditional artificial insemination methods), is much shorter, usually a two-three day window of time within the estrous period.[4]

Many breeders will note the swelling of the vulva and the serosanguinous discharge, and then count approximately nine days from this point and breed once or twice within a day or two. If the beginning of proestrus has been accurately determined, if the bitch has about a seven day proestrous period, if ovulation follows shortly thereafter, and if the stud dog's sperm is relatively long-lived, then this plan may likely result in a pregnancy. But if the bitch has a short proestrous period, or a very long one, the bitch will likely be bred too late or too soon, respectively, and be unlikely to conceive. Using the tools of vaginal cytology, vaginoscopy, and serum progesterone assays, a clinician can accurately determine the date of the luteinizing hormone (LH) surge, and further estimate dates for ovulation and the fertile period. Use and interpretation of these tools is relatively well known among veterinarians offering reproductive services for dogs. Much of this material has been recently reviewed.[3] This presentation will focus on explaining the physiological reasons behind the diagnostic tools of vaginal cytology, vaginoscopy, and serum progesterone monitoring.

Vaginal cytology

Endocrinology

All of the changes noted on vaginal cytology through the estrous cycle of the bitch are due to changes in estrogen concentrations in the body. Understanding how and why those changes occur and how their consequent effects appear as cytological changes observable in a clinical setting will help the clinician to better chart normal and abnormal cycles.

Estrogen is produced through a steroidogenic pathway starting in the thecal cells where various enzymes convert cholesterol into different pregnanes and eventually the androgen hormone androstenedione, which is then transported across the basement membrane into granulosa cells (Figure 1). The enzyme aromatase in the granulosa cells converts androstenedione into estrone and then another enzyme (17β-hydroxysteroid dehydrogenase type I) further converts estrone into estradiol. This steroidogenic pathway functions during active ovarian follicular activity.

During anestrus, the reproductive system of the bitch remains relatively quiescent, though pulsatile changes in gonadotropin releasing hormone (GnRH), follicle stimulating hormone (FSH), and LH in late anestrus are key to transitioning into proestrus.[5] Estrogen concentrations during anestrus are

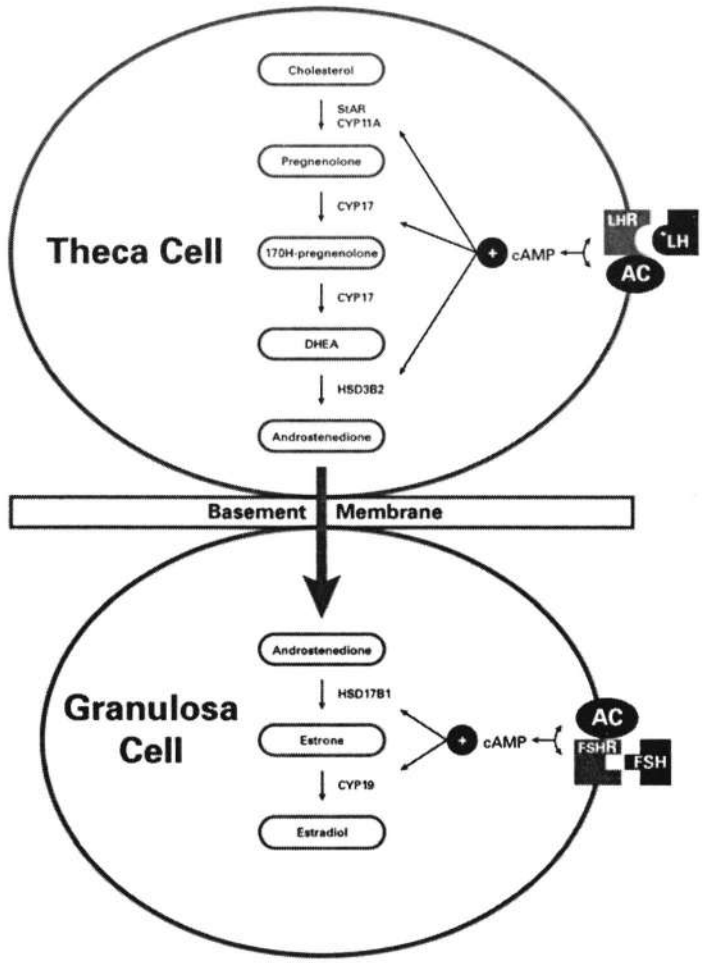

Figure 1. Follicular phase steroid biosynthesis in the ovary with the illustration of the two-cell/two-gonadotropin theory. Species variation in steroidogenic pathways exists within the ovary and the pathway provided in this figure is characteristic of human. AC, adenylate cyclase; FSH, follicle stimulating hormone; LH, luteinizing hormone; StAR, steroidogenic acute regulatory protein; CYP11A, cholesterol side-chain cleavage; CYP17, 17 α -hydroxylase,17,20-lyase; HSD3B2, 3 β -hydroxysteroid dehydrogenase type II; HSD17B1, 17 β - hydroxysteroid dehydrogenase type I; CYP19, aromatase.

mostly low, but some bursts of estrogen secretion may be noted and may coincide with minor follicular waves that consequently regress without maturing to a stage where ovulation might occur, though strong evidence for follicular development during anestrus is lacking.[6] At the end of anestrus, follicular development leads to rising estrogen concentrations (ranging from five to 15 pg/mL) as the follicular phase of proestrus begins.[5,7]

Estrogens dominate the bitch's endocrinological profile during proestrus, going from around 25 pg/mL in early proestrus to between 50 and 120 pg/mL in late proestrus.[6,8] Approximately 24 to 48 hours prior to the beginning of behavioral estrus and the onset of the LH surge, estrogen concentrations begin to drop.[8-10] One reason for this drop is that the follicular cells in the bitch experience preovulatory luteinization in which more and more follicular cells switch their biochemical pathways of steroidogenesis to produce progesterone instead of estrogen.[6,11] As a result, estrogen drops and progesterone rises. The sharp rise in estrogen concentrations, followed by the dual dynamic effect of dropping estrogen and rising progesterone concentrations likely cues both the beginning of behavioral

estrus and the LH surge.[11,12] The LH surge further stimulates ovulation, after which luteinization of the remaining follicular cells accelerates.[12] Progesterone concentrations will continue to rise and estrogen concentrations continue to fall throughout the remainder of estrus.[12] By the beginning of diestrus (metestrus), estrogen will be back to baseline concentrations (Figure 2).

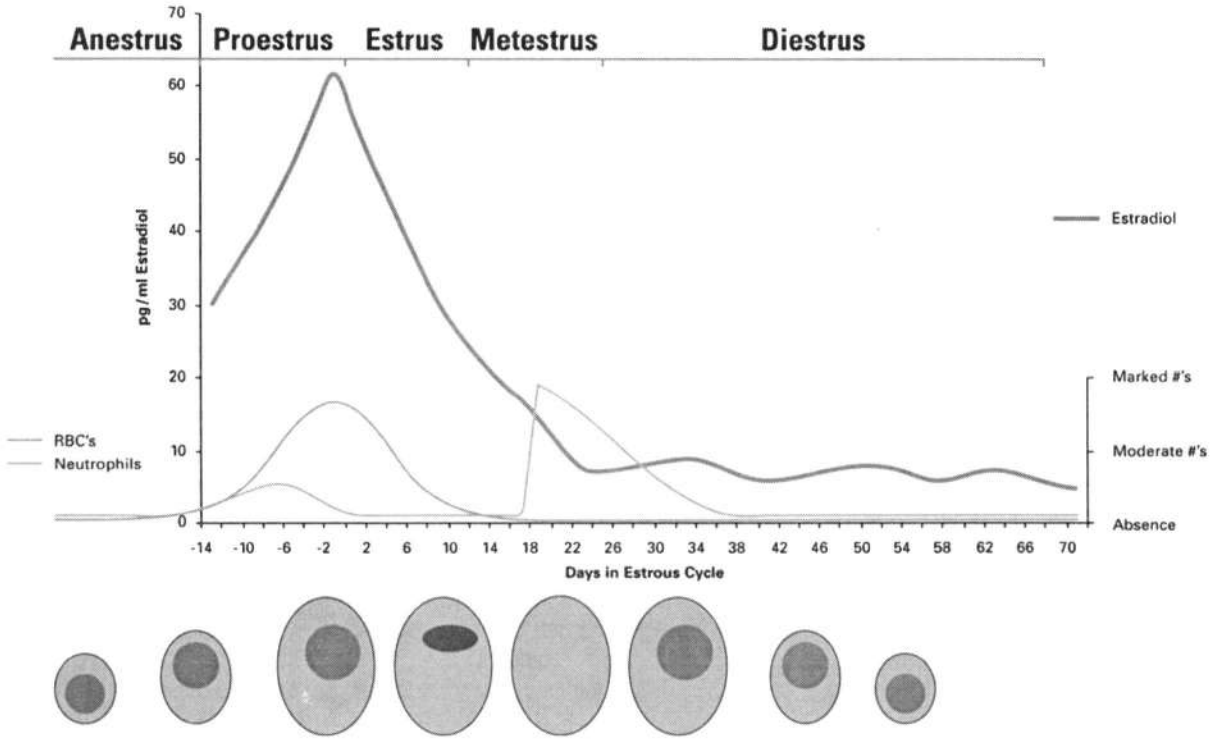

Figure 2. Relative abundance of red blood cells and neutrophils are depicted in comparison with serum estradiol concentrations and stage of the estrous cycle in the bitch. Also depicted are epithelial cell types, correlating with stages of the estrous cycle wherein they are dominant; from left to right: parabasal, small intermediate, large intermediate, superficial (with pyknotic nucleus), anuclear superficial cell, large intermediate, small intermediate, parabasal cell.

Vaginal epithelial cells

The vaginal epithelium is a very thin tissue layer in anestrus,[3] with only 1-2 layers of cuboidal epithelium.[13] Estrogen has a potent mitogenic effect on the cells of the vaginal epithelium, causing marked growth in the thickness of the vaginal wall as the epithelial cells multiply and the myometrium matures, forming prominent muscle bundles.[13] The capillary blood supply is located in the basement membrane and during anestrus even the cells farthest away from the capillaries are close enough for easy exchange of oxygen, nutrients, and waste products. During proestrus, however, as the vaginal epithelium drastically increases in thickness as cells multiply under the mitogenic influence of estrogen, the cells closer to the lumen are far enough away from the capillary bed that they are unable to either receive needed nutrients or to expel waste products. In such a state, the metabolic waste products build up in the cytoplasm of these cells, causing a dramatic increase in the cytoplasmic volume. This is manifest cytologically in the morphological differences in the progression of a parabasal cell to an intermediate cell to a superficial cell (Figure 2).

Eventually, due to a combination of exposure to cellular waste and lack of nutrient exchange, these cells keratinize and die. The nuclear chromatin condenses, causing the pyknotic appearance of some nuclei of superficial cells. The nuclear membrane then becomes disrupted and the nuclear material dissipates, leading to anuclear superficial cells, commonly referred to as "cornified" or "keratinized".

Red blood cells

Estrogen causes the uterine vasculature to become highly permeable and allow the leakage of red blood cells into the uterine lumen via diapedesis.[3] Estrogen also causes the relaxation of the cervix, allowing those red blood cells to exit the uterus and appear as the serosanguinous vulvar discharge noted typically during proestrus and early estrus in the bitch. The length of time that the discharge persists corresponds to estrogen exposure. In most bitches, estrogen returns to baseline by the end of estrus, and therefore the presence of red blood cells in the uterus and vagina wanes by this point (Figure 2). In some bitches, however, enough estrogen exposure remains into metestrus so that red blood cells may still be present at this stage. The presence of red blood cells indicates that the cervix is still relaxed and patent.

Bacteria

A normal vaginal flora exists and so there are bacteria present in the vagina at all stages of the estrous cycle. During proestrus and estrus, however, when secretions containing red blood cells are present, bacterial numbers increase markedly and often are noted in great numbers on vaginal cytology examinations during these stages of the bitch's cycle.

Neutrophils

Neutrophils are normally present in most of the tissues of the body in low numbers as they monitor the body for antigenic substances. As bacterial numbers increase during proestrus and epithelial cells begin to die and slough off, neutrophils increase in number in the vagina. Through chemotaxis, neutrophils are summoned to help clean up the bacteria and other cellular debris. Neutrophils access the vaginal mucosa via diapedesis. This migration is possible early in proestrus, when the vaginal mucosa is still relatively thin. As proestrus progresses, and the vaginal mucosa thickens, eventually the neutrophils are unable to travel the great distance from the capillary bed in the basement membrane to the vaginal lumen and the neutrophils become trapped in the thickened vaginal mucosa. When estrogen concentrations drop to baseline by the end of estrus, the thickened layers of the vaginal epithelium will slough off, releasing the trapped neutrophils along with the vaginal epithelial cells into the vaginal lumen, resulting in a dramatic increase in lumenal neutrophils for some days (Figure 2).

Vaginal cytological profiles by stage of estrous cycle

Cytological anestrus (Figure 3)

During anestrus estrogen is very low with short, sporadic increases in concentration. Lacking the mitogenic influence of estrogen, the vaginal mucosa does not increase in thickness and the vaginal epithelial cells remain close to their blood supply, living a relatively longer life than cells duirng proestrus that divide quickly and die quickly as they are far from their blood supply. As such, most of the epithelial cells seen on a vaginal cytology of an anestrous bitch are parabasal or small intermediate-type cells and are in much lower numbers than duirng other stages of the cycle when dead cells are sloughing off readily.[3] Since they tend to be older cells, their membranes are more fragile and they often appear as oblong or misshapen cells. They easily break and so "naked" nuclei with cytoplasmic streaming are common. Red blood cells are absent to rare since low estrogen levels do not cause increased permeability of uterine blood vessels during this stage. Neutrophils are seen only occasionally and usually not with intracellular bacteria. Mucus is a common finding and bacteria are present in low numbers.

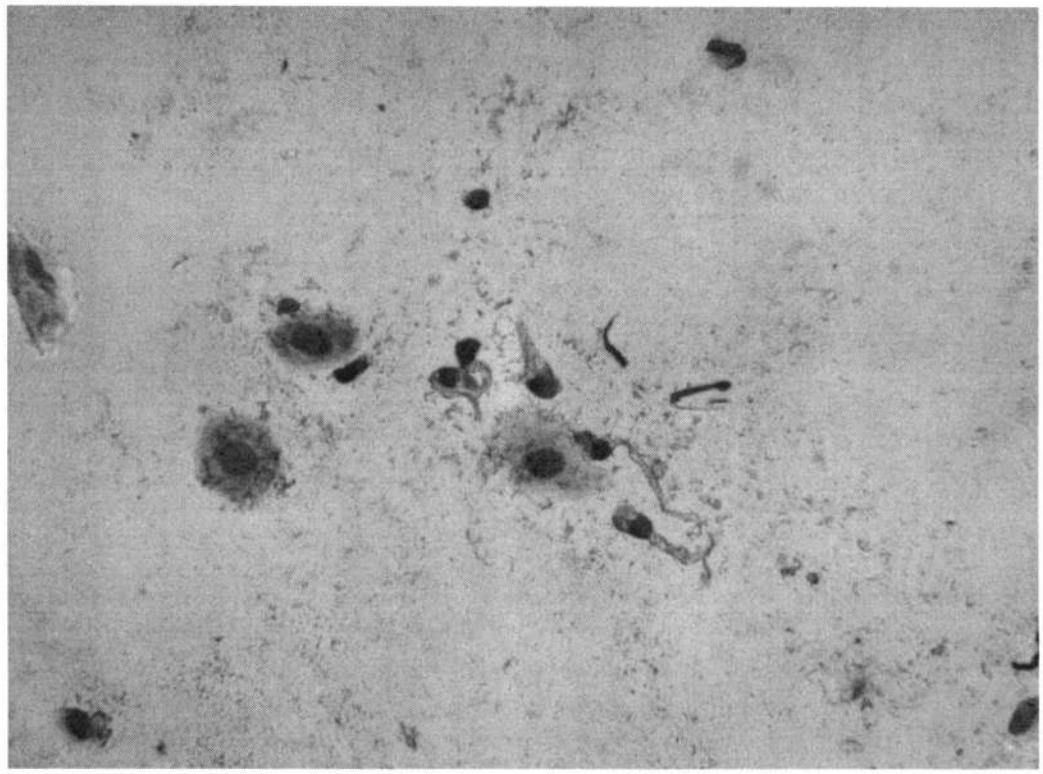

Figure 3. Anestrous cytology. Epithelial cells are parabasal or small intermediate. Some cells are misshapen. Broken cells show "naked" nuclei with cytoplasmic streaming. Mucus is present. Nuetrophils and red blood cells are absent (neutrophils may be seen on occasion in other fields).

Cytological proestrus (Figure 4)

In early proestrus, estrogen is rising quickly and is at peak levels towards the end of proestrus before beginning to fall as the bitch progresses into estrus. The thickening of the vaginal mucosa due to the mitogenic effect of estrogen causes increased distance between lumenal cells and the capillaries, which results in a build-up of cellular waste in the cytoplasm and a lack of nutrient and oxygen exchange. Large numbers of intermediate cells of varying sizes form initially and are the primary cells seen in early proestrus, mixed with parabasal cells and low numbers of superficial cells.[3] During mid- to late-proestrus, more and more superficial cells appear as large intermediate cells die and become superficial cells. Parabasal cells along with smaller intermediate cells disappear from the cytology. By the end of proestrus, the majority of the epithelial cells are superficial cells and this represents the eventual cytological transition into estrus. Due to the effect of estrogen on the uterine vasculature permeability and the softening of the cervix, red blood cells are usually present in large numbers throughout proestrus. Neutrophils are present in increased numbers in early- to mid-proestrus as they respond to the increased numbers of dead cells and bacteria, but neutrophils disappear from the cytology by mid- to late-proestrus as the vaginal mucosa becomes too thick for the neutrophils to traverse entirely and they become trapped in the lower levels of the thickened vaginal epithelium. Bacteria are usually present in very large numbers. Mucus is present early, but becomes less common in late proestrus.

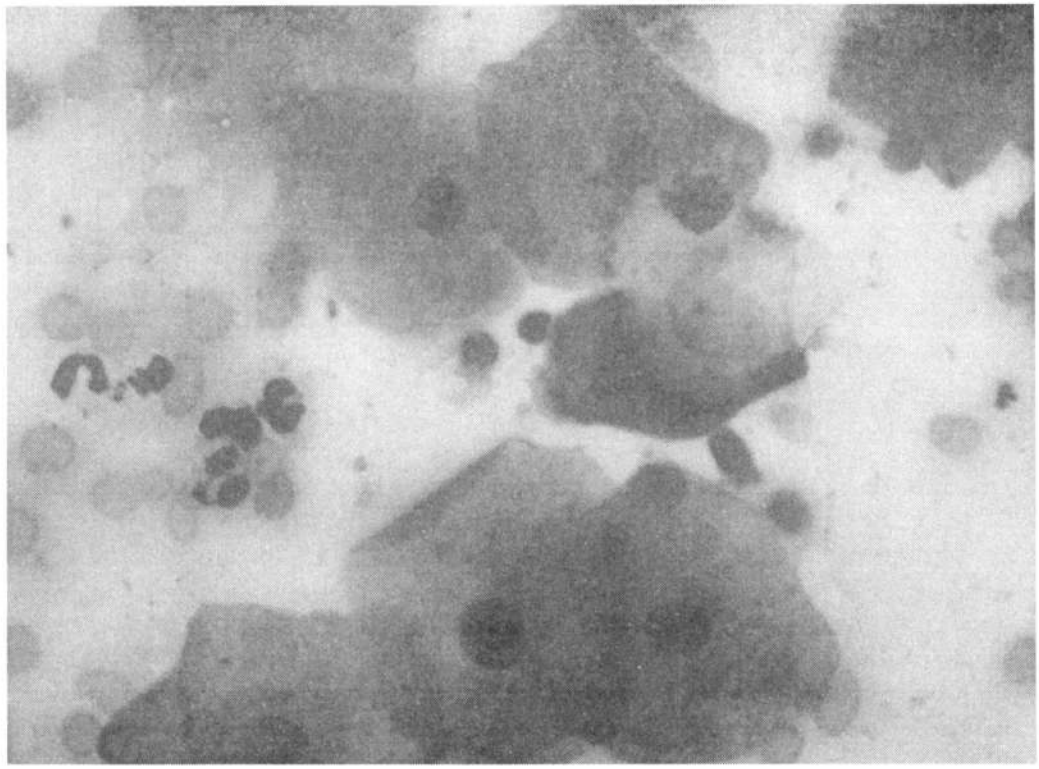

Figure 4. Proestrous cytology. Epithelial cells are predominantly middle or large intermediate cells. Some superficial cells are present. Red blood cells are abundant. Neutrophils are seen in low to moderate numbers.

Cytological estrus (Figure 5)

Estrogen concentrations are still relatively high in early estrus, but are falling. By the end of estrus, estrogen concentrations are back to baseline levels. Superficial cells in excess of 80% of total epithelial cells present defines the estrus cytology. The degree of cornification that occurs varies between individuals with some bitches showing 100% anuclear superficial cells by mid-estrus and other bitches maintaining a significant percentage of superficial cells with pyknotic nuclei and even a number of large intermediate cells (<20%) throughout estrus. Red blood cells are usually present in early estrus, due to relatively high concentrations of estrogen still present, but numbers of red blood cells tend to drop quickly as estrus progresses and usually disappear towards the end of estrus. Neutrophils disappear in mid-proestrus and are not seen during estrus. Bacteria may still be present, often in large numbers, during estrus. Mucus is usually not seen during estrus, creating a "clean" appearance to the background of the cytology slide.

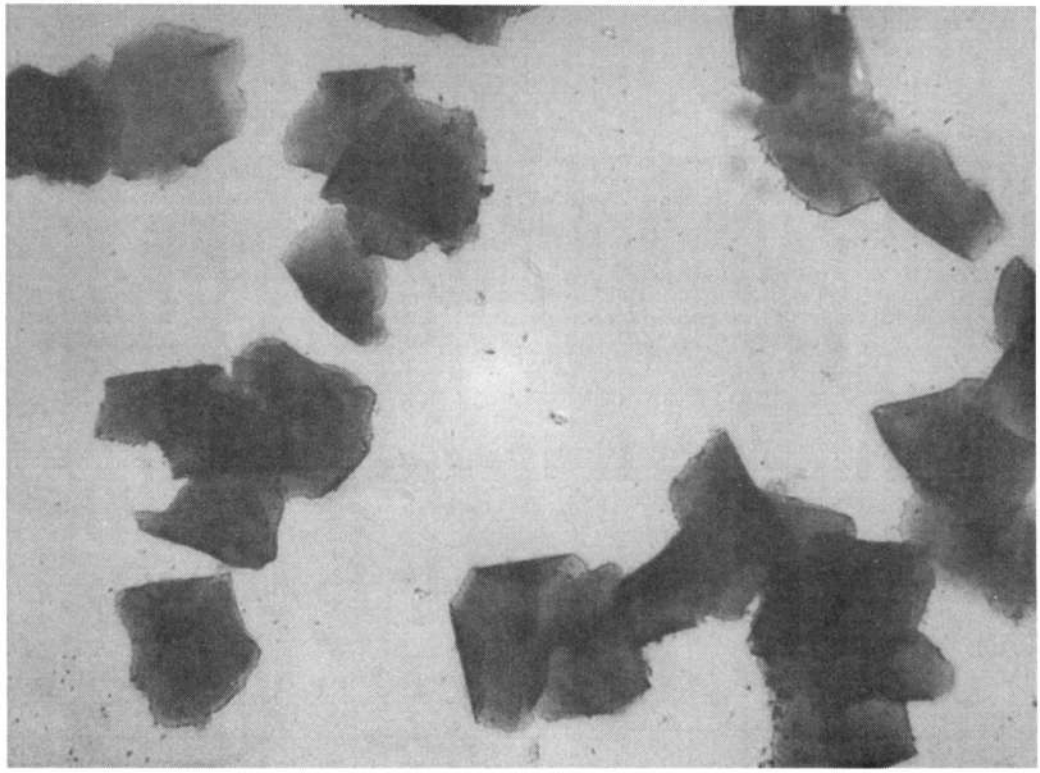

Figure 5. Estrous cytology. Epithelial cells are almost exclusively superficial cells. Red blood cells and neutrophils are absent (red blood cells may be seen in some estrous cytology evaluations). Mucus is absent.

Cytological metestrus (Figure 6)

Estrogen concentrations return to baseline as the bitch enters metestrus. With the mitogenic influence of estrogen gone, the vaginal mucosal cell layers rapidly slough off causing an infusion of large numbers of intermediate-type cells into the vaginal cytology. The transition from estrus to metestrus is defined specifically by the number of superficial cells dropping below 80% of the total vaginal epithelial cells and a predominance of different sizes of intermediate epithelial cells with some parabasal cells appearing. The speed of this transition is highly variable between individual bitches and may be gradual, taking days, or very rapid, happening overnight. It is common to see marked numbers of neutrophils during metestrus. This sudden appearance of neutrophils is due to the fact that neutrophils have been migrating towards the vaginal lumen, but unable to reach it due to the thickness of the tissue, since late proestrus and throughout estrus. These neutrophils have become trapped in the vaginal mucosa. As the cell layers of the vaginal mucosa slough off during metestrus, all of these neutrophils are released. This sudden rise in neutrophils and intermediate epithelial cells is called the "metestral rush". In addition to the large number of neutrophils typically seen during metestrus, some neutrophils somehow become engulfed within the cytoplasm of intermediate epithelial cells. This cellular anomaly has been called a "metestral cell" and is typically only seen during metestrus. While an increased number of neutrophils is typical of metestrus, it should be noted that individual variation exists and some bitches do not show a return of neutrophils in early metestrus. In these bitches, the switch from >80% superficial cells to <80% superficial cells will be the only change noticed between estrus and metestrus. Red blood cells typically wane and disappear by the middle or end of estrus, as estrogen concentrations fall, but occasional bitches will have sufficiently elevated estrogen concentrations during late estrus and early metestrus to still affect some vascular permeability, resulting in red blood cells in metestral cytology. Bacteria are usually still present during metestrus, though they tend to decrease in numbers as red blood cells decrease. Mucus is often still absent during metestrus, though it may return after a few days.

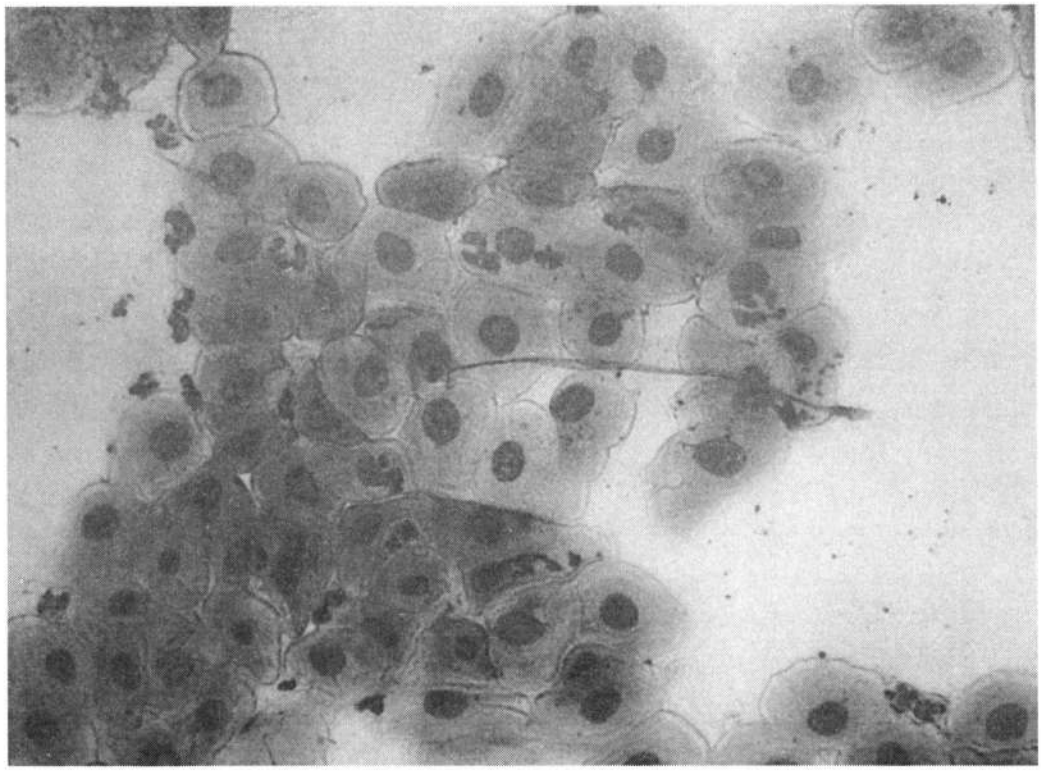

Figure 6. Metestrous cytology. Epithelial cells are intermediate cells predominantly, of all sizes. Some superficial and parabasal cells may be present. Neutrophils are abundant (not always, but often, the case). Red blood cells are absent.

Cytological diestrus (Figure 7)

Estrogen concentrations will have been at baseline for many days and the transition from the metestral to the diestral smear is gradual, but the eventual cytological picture is very different in mid- to late-diestrus from early metestrus. Superficial and large intermediate cells disappear, and the predominant epithelial cells are middle and small intermediate cells and parabasal cells. Neutrophils decrease in number. Red blood cells, if they were present at all in metestrus (uncommon), should be entirely gone by diestrus. Bacteria are present, but in lower numbers than seen in proestrus, estrus, and possibly metestrus. Mucus returns to the smear. As diestrus progresses, the cytology will begin to look more and more like anestrus, with cell numbers decreasing and consisting of mostly parabasal and small intermediate epithelial cells with an occasional neutrophil observed.

Figure 7. Diestrous cytology. Epithelial cells are predominantly middle and small intermediate cells and parabasal cells. Neutrophils may be abundant (as in this smear). Red blood cells are absent. Mucus returns.

Vaginoscopy

Whereas changes in vaginal cytology reflect only the effects of estrogen, vaginoscopic evaluation of the bitch allows the practitioner to track the effects of both estrogen *and* progesterone. Initially, the changes noted are similarly reflective of changes in estrogen. During anestrus, when estrogen is very low and the vaginal mucosa consequently very thin and very close to the capillaries, vaginoscopic evaluation of the bitch reveals a very smooth, pink lumenal wall (Figure 8). The only fold is the dorsomedial fold, which is a permanent anatomical structure. Estrogen has a local vasoconstrictive effect in the vaginal area, causing an increase in edema in the vagina.[13] This, along with the mitogenic effect discussed earlier, results in a rapid increase in the vaginal mucosal depth. The cells of the lumen move farther away from their capillaries, and the color of the lumen consequently changes eventually from pink to pale. The vaginal edema manifests as folds of tissue. Initially, early in proestrus, these folds run parallel to the lumenal tract, in a longitudinal fashion, and are called primary folds (Figure 9). At this point the mucosa is still pink. As the estrogen levels continue to rise in proestrus and edema increases, transverse folds appear on top of the primary folds, and these are termed secondary folds.[14] The mucosa at this stage begins to be more pale (Figure 10). All these changes are, once again, due to the influence of estrogen alone. But as progesterone begins to rise late in proestrus, it relaxes the vasoconstrictive effect of estrogen. The result is a resolution of the vaginal edema. The primary and secondary folds shrivel as they, in essence, dehydrate. The resultant effect is the very wrinkled appearance of crenulations that are noted duirng estrus (Figure 11). These crenulations are very pale in color. Adding vaginoscopy to the evaluation and noting when crenulations begin gives the practitioner instant information that progesterone concentrations are rising.

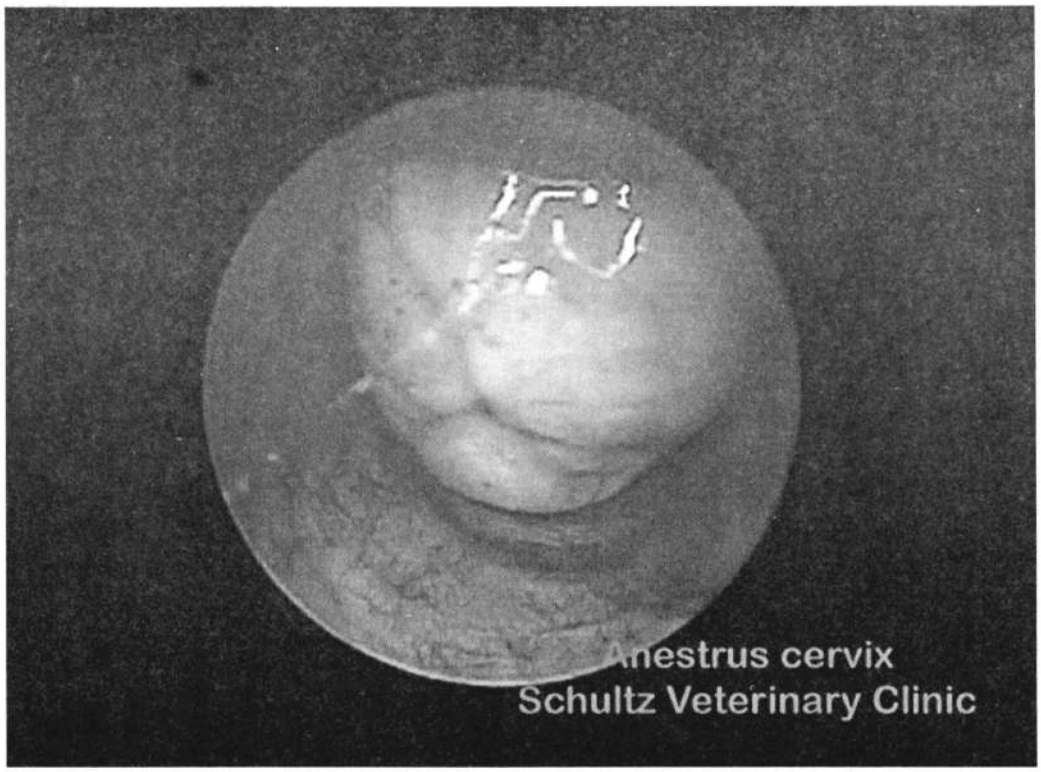

Figure 8. Anestrous vaginoscopy. Vaginal mucosa is smooth and pink. The cervix is pictured here. The dorsomedial fold (not pictured here) may be seen, but other folds are absent. Photo courtesy of Dr. Will Schultz.

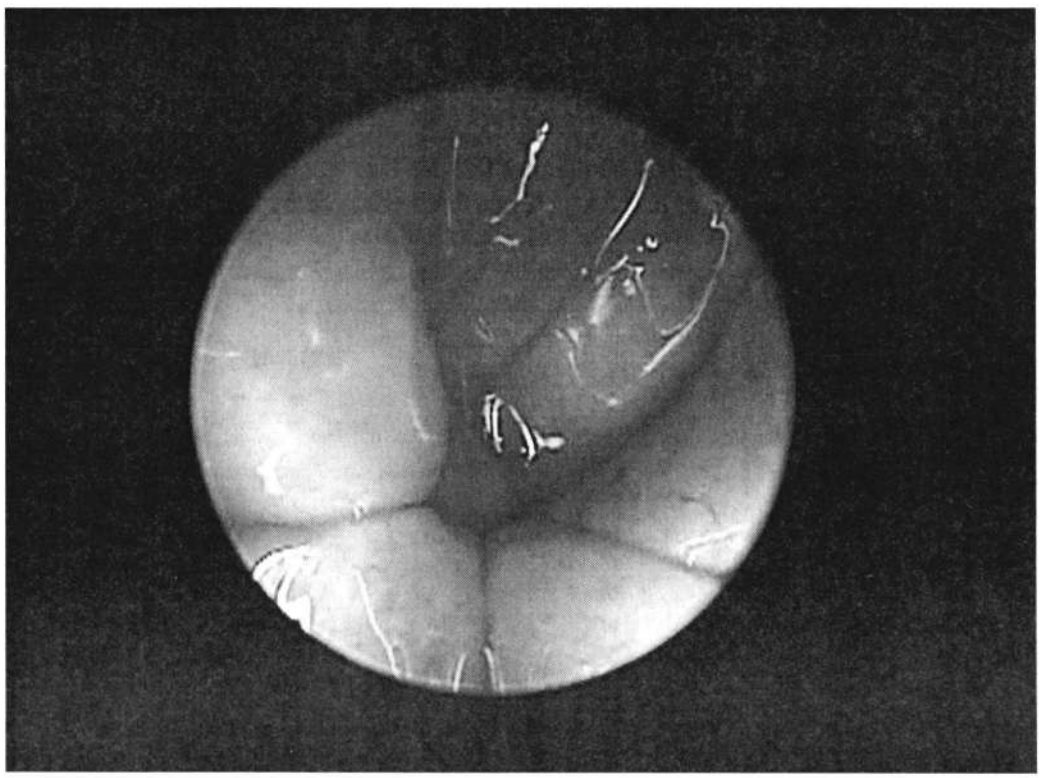

Figure 9. Early proestrous vaginoscopy. Vaginal mucosa is pink and has primary folds, which run longitudinally along the vagina. Photo courtesy of Dr. Will Schultz.

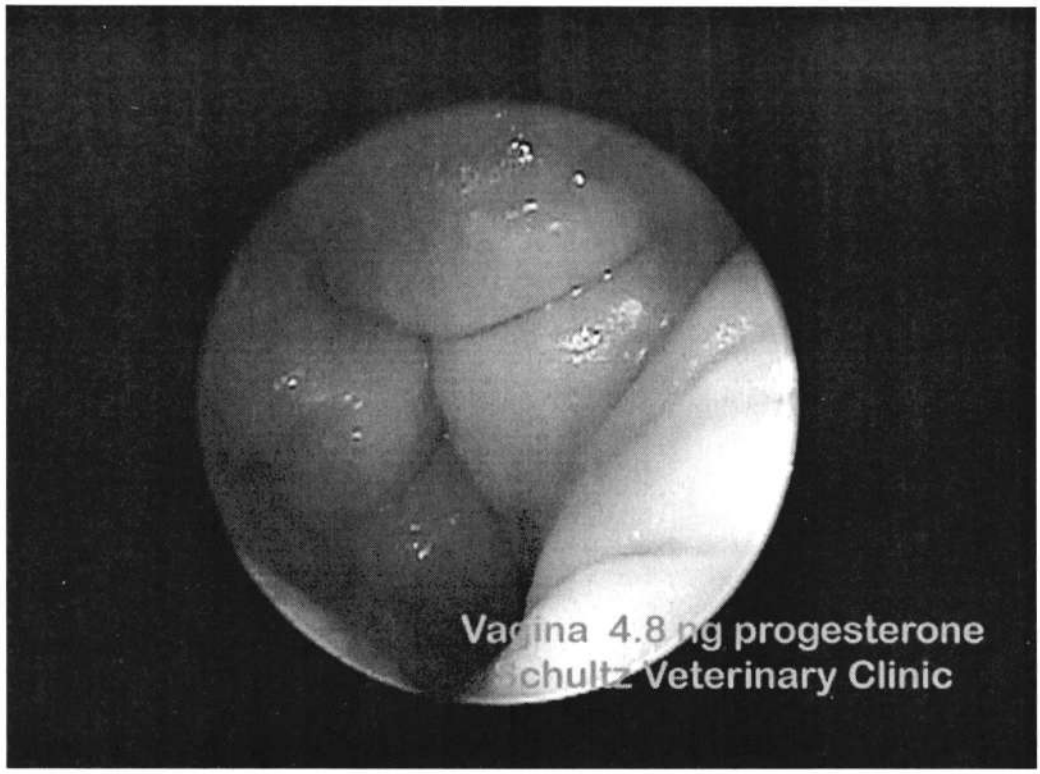

Figure 10. Late proestrus vaginoscopy. Vaginal mucosa becomes paler and secondary folds, transverse to the primary folds, form. Photo courtesy of Dr. Will Schultz.

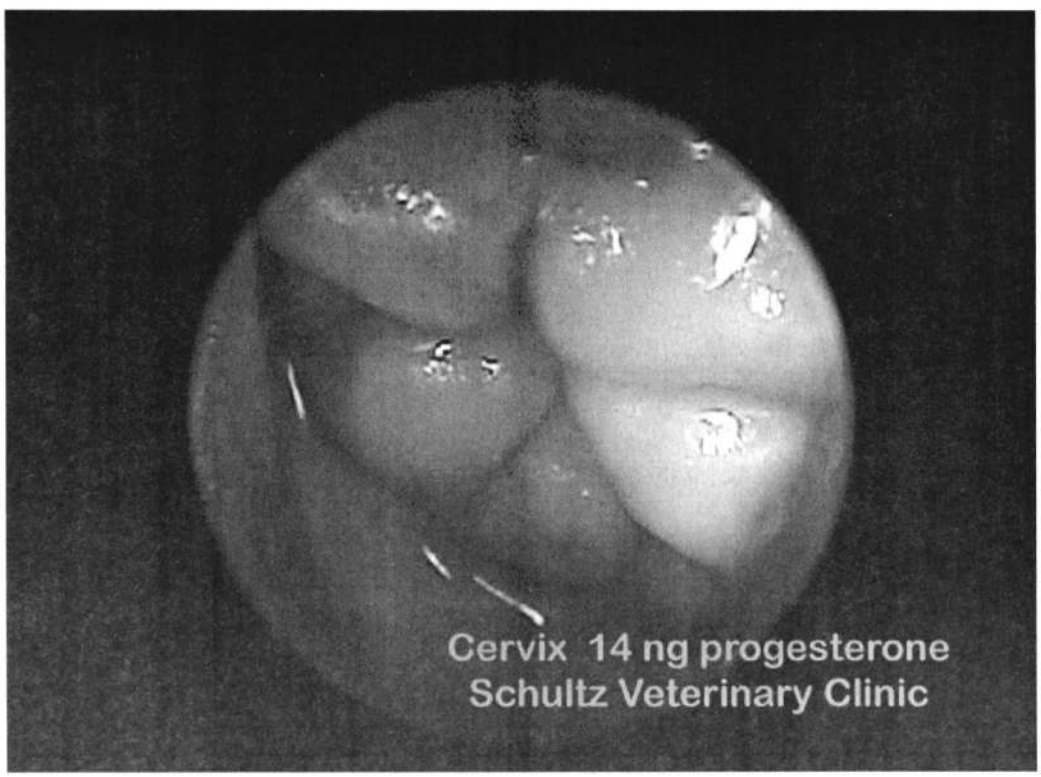

Figure 11. Estrous vaginoscopy. Vaginal mucosal folds shrink and wrinkle, which are called "crenulations." Mucosa is very pale. Photo courtesy of Dr. Will Schultz.

Serum progesterone assay

Progesterone in the bitch comes primarily from luteal tissue in the ovaries. During anestrus, baseline serum progesterone concentrations are typically less than one ng/mL. During proestrus, as discussed above, preovulatory luteinization occurs in some of the follicular cells, causing them to switch from estrogen to progesterone production days prior to ovulation.[6] Serum progesterone concentrations during proestrus climb slowly to around one ng/mL, where they will linger for a few days to a couple weeks.[15] At some point, the concentrations will begin to rise again and will reach around five ng/mL before the bitch ovulates. Once this rise in progesterone starts, the rate at which the progesterone concentrations rise is relatively consistent among most individuals and thus the clinician can correlate the physiological events of the LH surge, ovulation, and the start of the fertile period with subsequent concentrations of serum progesterone (Figure 12). The LH surge typically lasts between 24 and 36 hours in the bitch and begins when the serum progesterone concentration has shown an abrupt rise from < 0.5 ng/mL to around one ng/mL.[3,12] Peak LH surge levels coincide with serum progesterone concentrations around two ng/mL. For accurate chronology of ensuing events, the LH surge is considered day zero. After the LH surge, it usually takes approximately two days before ovulation occurs (i.e. ovulation occurs at day two),[3,15] though the range of time from the LH surge until ovulation is from one to four days.[16] At the time of ovulation, the serum progesterone concentration is usually > five ng/mL.[3] Because of the variability in time between LH surge and ovulation, noting the abrupt rise in progesterone to around five ng/mL is a more reliable indicator of ovulation than measuring the LH surge alone.[12]

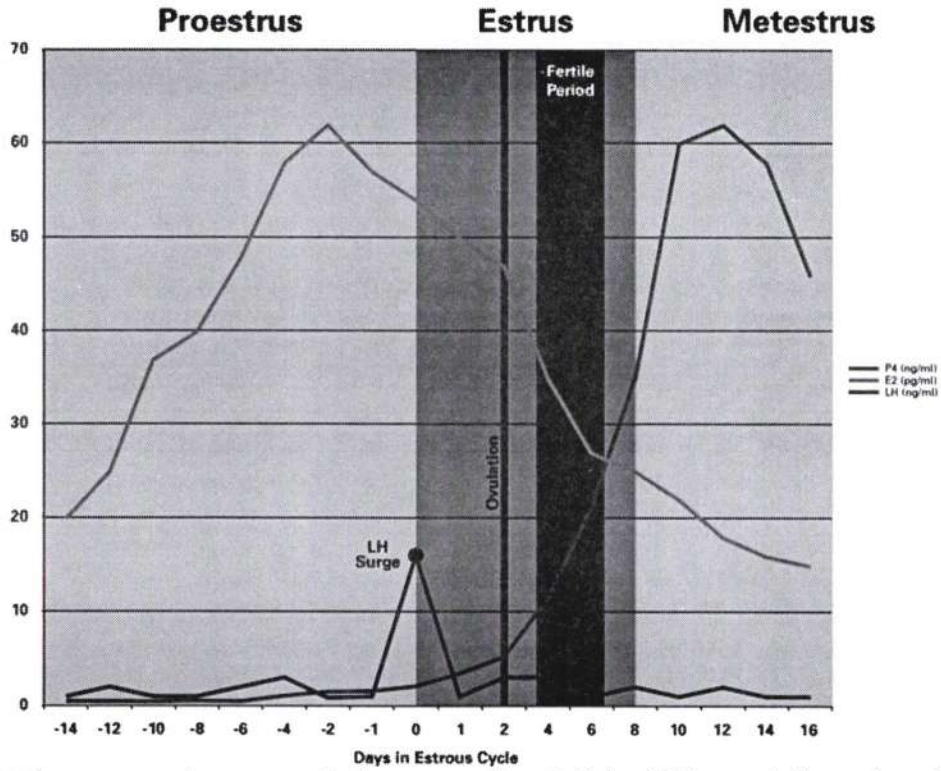

Figure 12. Changes in serum progesterone concentrations compared to estradiol and LH concentrations and correlated with key events: LH surge, ovulation, and the fertile period of the bitch.

In all other domestic species, the oocyte is fertile immediately after ovulation. This is not the case in the bitch. In the bitch, the oocyte is not fertile until usually one to two days after ovulation (i.e. the fertile period starts around day four).[17] At this point, serum progesterone concentrations have often climbed to between six and ten ng/mL.[14]

To understand why there is a two-day delay in fertility after ovulation in the bitch, the process of meiosis must be reviewed (Figure 13). During meiosis, primordial germ cells (diploid) progress through mitosis to form oogonia (also diploid), which divide via mitosis to form primary oocytes (still diploid). Meiotic prophase then causes these diploid (2N) cells to replicate, but stops partway through the meiotic process, when the cells are at a 4N stage. The primary oocytes within dominant follicles are arrested at this stage (indeed, it is called "nuclear arrest") until the LH surge, which removes the meiotic inhibitors, and the primary oocytes extrude the first polar body and become diploid (2N) again. At this stage they are called secondary oocytes. In all other species except the bitch, this is the stage of ovulation, and the second polar body is extruded, bringing the secondary oocyte to a haploid (1N) state, ready for fertilization by the sperm (also haploid, 1N). Their fusion forms a zygote (2N) and continues through normal embryonic divisions and development. The bitch is unique among domestic species in that she ovulates a primary oocyte, which still must complete meiosis before it is a secondary oocyte, ready for fusion with the sperm cell.[18] This process takes about two days to complete and therefore the need to wait an additional two days after ovulation before the canine oocytes are fertile.[19]

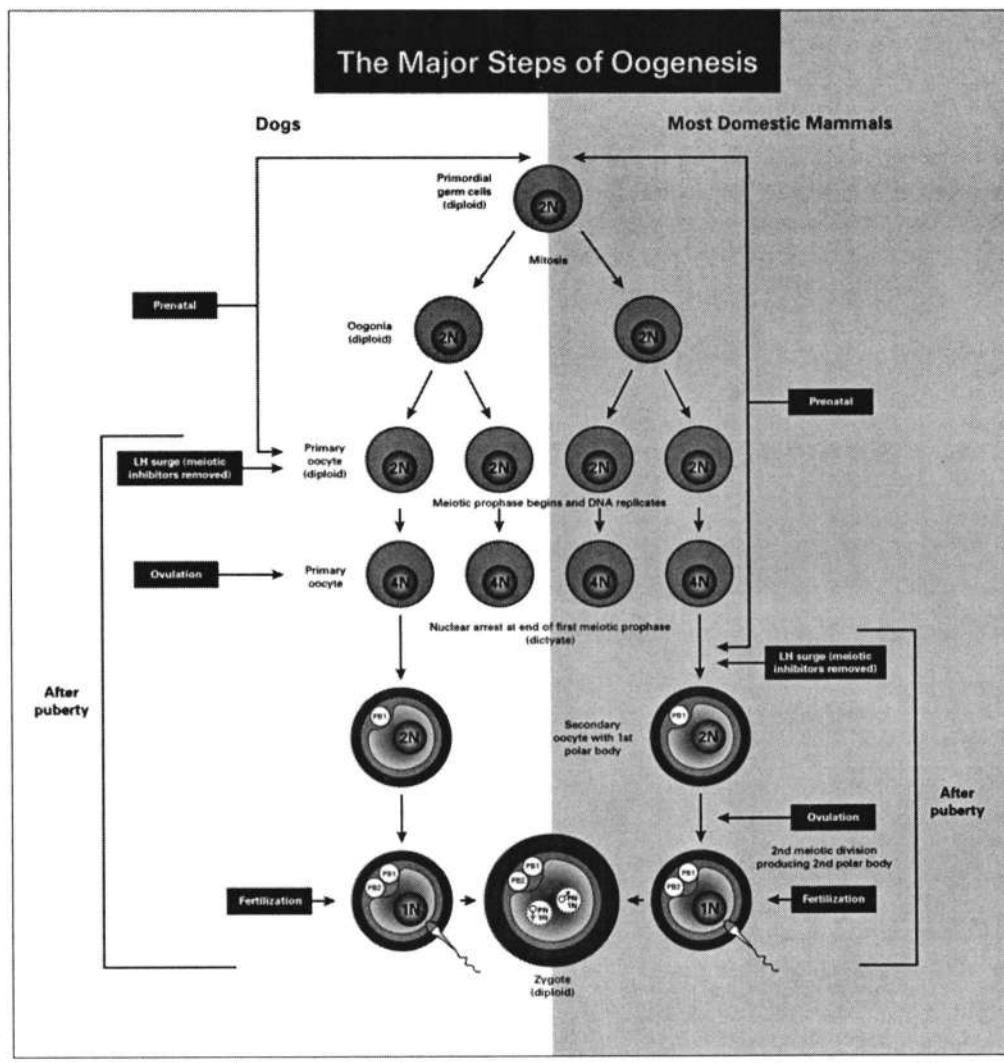

Figure 13. Comparison between major steps in meiosis between most mammals and the bitch. Primary differences include the ovulation of a primary oocyte instead of a secondary oocyte in the bitch, requiring time for the ovulated oocyte to mature before it is ready for fusion with the sperm cell. Figure modified from PL Senger: The follicular phase. In: Pathways to pregnancy and parturition. Pullman (WA): Current Conceptions, Inc; 2003. p. 182. Used with permission.

Often bitches may be bred a few days before the recognized start of the fertile period and still become pregnant. This is possible because the sperm from fertile male dogs usually remain viable inside the female tract for at least a couple of days and in some cases up to a week.[20] On the other side of the equation, once the oocyte matures to a secondary oocyte, it likewise maintains its fertility for at least a couple of days (up to over 200 hours after ovulation, in some cases).[4] In fact, studies have shown that it is usually not the oocyte losing fertility that causes a cessation to the fertile period, but rather the fact that rising progesterone concentrations cause the cervix to close,[4] thus effectively shutting off access for vaginally deposited sperm to reach otherwise fertile oocytes in bitches bred longer than two to four days after the start of the fertile period (i.e. those bred after days six to eight, varying by individuals). Serum progesterone concentrations are usually around 22.5 ng/mL (± 3.4 ng/mL) at the end of the fertile period.[4]

This end to the fertile period, however, is most applicable to natural mating and vaginal artificial insemination methods, since these methods deposit the semen in the cranial vagina, for which a closed cervix would be an obstacle. Any method that deposits the semen into the uterus directly, such as transcervical insemination (TCI) or surgical insemination, can extend this fertile period for another couple of days.[4] Anecdotally there are many reports of successful inseminations using TCI or surgical insemination when progesterone concentrations were up to 25 ng/mL, and some reports of even higher, though fertility seems to wane after that.

The timeline outlined in Figure 12 pertains to the average cycle. Not every bitch in every cycle will follow this exact pattern. It is beyond the scope of this presentation to cover such events as delayed ovulation and split heats. It is worth pointing out, however, that it is a responsible and wise decision to monitor progesterone until at least ovulation, or ideally until the fertile period is reached. This will ensure that individual variation between bitches and cycles is noted and appropriate compensations are made. In some cycles, the progesterone concentrations will rise faster than the timeline in Figure 12, and insemination will need to be done sooner than anticipated. Other cycles will demonstrate that the progesterone lingers around two ng/mL for many days before climbing, indicating that ovulation is delayed longer than anticipated and insemination should be delayed accordingly.

Summary

The diagnostic tools used for breeding management in the bitch, vaginal cytology, vaginoscopy, and serum progesterone concentrations, are widely used. Understanding the endocrinology and physiology behind these tools helps to better interpret both normal and abnormal cycles.

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Small animals' advanced reproductive techniques - embryo collection

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Abstract

Methods for successful embryo retrieval are needed for bitches and queens incapable of carrying a pregnancy to term, for cryopreservation of genetic material, for the production of embryonic stem cells or for studying normal embryology. Methods for recovery of canine embryos and various factors affecting their number, developmental stage and quality are discussed. A brief summary of published reports regarding collection of feline embryos is included.

Keywords: Embryo collection, embryo transfer, cryopreservation, embryonic stem cells

Introduction

Reasons for the collection of preimplantation embryos from bitches include the study of normal embryonic development in the bitch with a view to better understanding the normal physiology of ovulation, fertilization, hatching, migration and implantation; for transfer or cryopreservation in valuable bitches with uterine pathology or a history of infertility or resorption or in working dogs too busy or too old to whelp and raise puppies; to make embryonic stem cells to treat conditions in dogs; or as models for human stem cell research.[1-9] Dogs are used frequently as a model for studying human diseases, therapies and conditions. Several hundred genetic diseases of dogs mimic similar human conditions. Thus, dogs are common inhabitants of research facilities around the world. Dog breeds encompass those that resemble their wild ancestors, such as Northern breeds, and those that are barely recognizable as dogs such as English Bulldogs. Dogs range in size from Yorkshire terriers to English Mastiffs. Most of the basic reproductive physiology we know comes from research performed on Beagles and occasionally Labrador retrievers[9,10] or hounds[5,6,11] or mixed small breeds.[12]

Currently, much interest lies in obtaining embryonic stem cells from all species for therapeutic purposes. Naturally the canine embryo is being evaluated as a source of embryonic stem cells for therapies in dogs and also with a view to studying their therapeutic potential in human conditions.

Early embryology

Embryos reside in the canine uterine tubes for much longer after ovulation than those in other domestic species, probably because they require the first 60 hours in the uterine tube to become fertilizable. Morulae enter the uterine horn 8.5 days after ovulation (10.5 days after the luteinizing hormone [LH] surge)[2] or by 8.5 to 10 days after ovulation (11 to 12 days after the LH surge).[10, 13-18] The fat-filled bursa which completely envelopes the canine ovary makes manipulation of the uterine tube difficult *in situ*. Identifying the uterine tubes proves difficult even after ovariohysterectomy. The fimbria can be found at the slit-like bursal opening, but the fat lining the bursa often obscures the rest of the uterine tube. Thus, it is much easier to flush embryos from the uterine horns than from the uterine tubes, both *in situ* and *ex* vivo.[19, 20] Also, a higher percentage of embryos per corpus luteum are recovered from the horns than from the tubes.[19] Furthermore, some reports show that blastocysts are more likely to result in a puppy than are early morulae.[21] Embryos from the uterine tubes may be transferred to the uterine horns, but survival is poor.[3] When flushing uterine tubes, it is necessary to flush from the fimbria toward the uterine body as fluid will not travel through the uterotubal junction in the opposite direction. Thus, the only possibility for non-surgical embryo collection would be those already located in the uterine horns. Morulae or blastocysts are typically transferred in livestock species.[22] Lastly, chances of surgical complications such as hemorrhage will likely be lessened further into diestrus. For all these reasons, flushing the uterine horns and body after the embryos have passed through the uterotubal junction is the preferred method to retrieve embryos.

Several criteria are used to attempt to collect blastocysts or morulae. The most important is ovulation timing as it is essential to be late enough to have uterine embryos and early enough to flush prior to the embryos hatching from the zonae pellucidae or implanting.

Embryo flushing–uterine tubes

Nonetheless, if one desires tubal stage embryos, uterine tubes may be flushed less than 11 days after the LH surge. In one study[23] a special 6 ga bulbed needle was used. In another study[24] a flanged intramedic catheter, outside diameter 1.27 to 1.57 mm (Becton Dickinson, Franklin Lakes, NJ) was placed into the fimbria. The bulb or flange was maintained in the proper location by placing a suture around the uterine tube. Hams' F10 (4 to 10 mL); Minimal Essential Medium (MEM); modified Dulbecco's phosphate buffered saline (PBS); or TCM 199 supplemented with HEPES have been used to flush uterine tubes. Hossein et al[23] inserted a 23 to 27 ga needle into the tubal lumen near the uterotubal junction for fluid collection. Other authors have collected the fluid from the uterine horn near the bifurcation similar to the following procedure for flushing uterine horns only.

Embryo flushing–uterine horns

The reproductive tract is often quite friable and hemorrhages easily in early diestrus. Although it is easier to spay the bitch prior to flushing out the embryos, several authors have flushed *in situ* in order to maintain breeding capability.[12,25] Surgery should be performed 11 to 15 days after either a measured or estimated LH surge to retrieve uterine embryos prior to hatching.[26] A midline ventral abdominal incision is made, the uterine body and one uterine horn exposed. Umbilical tape is placed distal to the uterine bifurcation across the uterine horn not currently being flushed to prevent loss of fluid up that horn. A 20 ga 3.75 cm needle with the tip broken off using a hemostat is used to make a hole into the lumen of the uterine body. An in vitro fertilization catheter (Kendall Sovereign IVF catheter, Tyco Healthcare Group LP, Mansfield, MA) is inserted for a short way (approximately 1.5 cm) up the uterine horn and digital pressure is maintained around the horn and catheter at that area to attempt to force fluid to escape only through the catheter. The same blunted needle attached to a 10 mL syringe (Norm-Ject Latex and silicone oil free syringe, Henke Sass Wolf GMBH, Tuttlingen, Germany) full of flush medium (ViGRO ™ Complete Flush, originally from AB Technology and then Bioniche Animal Health USA, Inc., Pullman, WA) is then used to penetrate the lumen of the uterine horn near the uterotubal junction. Fluid is pushed out of the syringe toward the bifurcation and collected into a sterile polystyrene 100x15 mm Petri dish (Fisherbrand, Fisher Scientific, Ottawa, ON, Canada) or a sterile urine collection cup (Sterile 118 mL Specimen Container, Fisherbrand, Fisher Scientific). The procedure is then repeated on the opposite horn.

When an ovariohysterectomy is performed, the reproductive tract is maintained at approximately 36°C until the embryo flushing procedure begins (15 min to 1 hr). The uterus should be patted dry of any excess blood, and placed inside a laminar flow hood (if available) in a room with ambient temperature 26.5 ± 1°C. A curved hemostat is placed on the horn that is not to be flushed, near the bifurcation. This prevents flush medium from going up the opposite horn rather than exiting through the uterine body and also allows one to hold the uterus vertically while flushing. An IVF catheter is inserted into the opening into the uterine body or horn and ensures a patent lumen into the first uterine horn to be flushed. The catheter is then removed and flushed with flush medium. A blunted 20 ga needle (see above) is placed into the distal horn as close to the uterotubal junction as possible. Flush medium is injected through the needle using a full 10 mL syringe (as above). The uterus is held over a 100x15 mm polystyrene Petri dish and the fluid collected. The process is repeated on the other horn, flushing into a different Petri dish. This enables one to see if all the possible embryos are collected from that horn. Liu et al[27] described a method to flush the uterus whereby they catheterized the tip of one horn and collected the fluid after it had gone through the body and up the other horn. The process was then repeated sending fluid in the opposite direction. Whatever method is used to flush, there are several ways to more easily dislodge embryos stuck deep in uterine folds such as filling the horn and milking it, causing turbulence in the lumen by following fluid with air or forcing fluid in rapidly then slowly.

Embryos are identified, preferably with a dissecting microscope (Wild M8, Wild Leitz Canada, Ltd., St-Laurent, PQ, Canada) and promptly removed from the Petri dishes and placed into holding medium (ViGRO™ Holding Plus, AB Technology) until further use. One can use a "normal" microscope on 4X or 10X, but a dissecting microscope is preferable to assess the stage and quality of the embryos. One should examine the whole Petri dish for small (120 to 200 μm diameter) discreet dark objects with a clear halo (zona pellucida) surrounding them. An embryo will roll whereas bits of debris or endometrium are less likely to be spherical. There are several ways to ensure that you have searched the whole dish, the most common to scratch some sort of pattern, either a spiral or a grid, on the outside bottom of the dish. If a lot of blood is in the fluid, light is difficult to shine through it and so one may either dilute the blood with more flush medium and place half in another dish or decrease the depth of fluid in the dish.

Embryo handling

Once found, embryos are removed from the collecting dish and placed into a holding medium of some sort, usually the same as the flush medium but with added serum or bovine serum albumin (BSA). Increasing the serum or BSA in the medium makes the embryos less likely to stick to the endometrium or the holding dish. Blood clots will attract and hold canine embryos and despite aggressively knocking the clot around in the dish, these may have to be removed using a needle or scalpel to cut the clot apart. The embryonic stage and quality should be characterized.[28] To do this the embryos should be rolled over and over in the dish with an IVF catheter or syringe and needle. If flushing between 11 and 15 days after the LH surge, one should find morulae, early blastocysts, blastocysts, expanded blastocysts, and occasionally hatching or hatched blastocysts. The greatest difficulty is determining whether a nicely compacted morula is a morula or an oocyte. The vitelline membrane should be obvious in an oocyte whereas the edge of the compact morula should be obviously scalloped due to the individual blastomeres making up the morula (mulberry).

Corpora lutea numbers may be estimated by palpating the ovary through the bursa during the *in situ* flush. After ovariohysterectomy, the bursal opening is enlarged in order to view the ovary. Corpora lutea numbers can still only be estimated. Careful sectioning of the presumed corpora lutea enables identification of the irregular, somewhat triangular shaped fluid core of each corpus luteum. The final accepted number of corpora lutea is determined by the number of these irregularly shaped cores found per ovary or where a distinct line demarcates two corpora lutea. Considering the difficulty in counting corpora lutea, one would be prudent in looking at corpora lutea numbers as plus or minus one per bitch, especially during *in situ* flushes.

Uterine horns may be flushed several more times if fewer embryos than corpora lutea are recovered. Uterine tubes may be flushed if neither embryos nor oocytes are recovered. An IVF catheter is placed into the tube through the fimbria, after exposing the ovary. The uterine tubes are flushed in the same direction as the uterine flushes with fluid being collected similarly near the uterine bifurcation.

The advantages of flushing the tract *ex vivo* include: embryos can be located and transferred to other media while another operator closes the abdomen; the ovaries can be properly examined to get a more accurate count of corpora lutea; uterine horns and/or tubes can be flushed multiple times trying to find embryos; anesthesia time is shortened; loss of the fluid carrying the embryos is less likely; the tract can be opened completely for examination; research bitches are now spayed and ready for adoption; flushing *ex vivo* results in a higher yield of embryos than flushing *in situ*[25] or similar percentages.[12] The advantages of flushing the uterus *in situ* are primarily that a valuable bitch remains available for further embryo retrievals. Unfortunately, so few embryo collections have been performed *in situ*, that the risk of damage to the reproductive tract and any possible negative effects on future fertility or embryo collections are unknown.

The number of embryos recovered per bitch depends on the percent recovery relative to corpora lutea present; the number of corpora lutea; the size of the bitch; the method of flushing – *in situ* generally less than *ex vivo*; the bitch's age; breeding factors such as semen quality and timing of insemination; pathology of the ovaries or uterus; and unknown factors.

Embryo transfer

Very few canine embryos have been transferred.[9,15,21,25] All reported transfers have been performed surgically except for the cryopreserved embryos mentioned below.[9] Of the reported transfers, some worked after transferring hatched blastocysts only[21] and others from morulae[15] or mostly morulae but a few blastocysts[25] or uterine tube stage embryos to uterine horns.[3] Most used an 18 or 20 ga needle to transfer a minimal volume of fluid near the uterotubal junction into either one or both horns.

If one could cryopreserve canine embryos, it would enable many more embryo transfers to be performed as one would not have to synchronize ovulation in the donor and recipient bitch. Recently, successful cryopreservation via vitrification of canine 4-cell to morula stage embryos has been reported.[9] Impressively, these embryos, once thawed, were transferred transcervically using an endoscope and a 5 to 8 Fr catheter. The transfer of 77 embryos to 9 bitches resulted in 4 pregnancies the birth of 7 puppies. Unfortunately blastocysts did not usually survive the vitrification and thawing procedures.

Feline embryo collection

By six to eight days after either natural mating or human chorionic gonadotropin (hCG) injection and artificial insemination, feline embryos will be in the uterine horns.[29,30] Surgical feline embryo collection has been described by Goodrowe et al.[31] They made a routine midline abdominal incision, punctured the uterus into the uterine lumen just cranial to the bifurcation and directed a tom cat catheter slightly up one horn. A 17 ga catheter was inserted into the same horn as near the uterotubal junction as possible and 25 to 30 mL of Ham's F10 medium with 5% fetal calf serum was used to flush the embryos out of each horn. Tsutsui[29,30] used 3 to 4 mL of Ringer's solution with 20% feline serum for each horn, flushing from the uterotubal junction as well, but fluid was collected through an 18 ga needle near the base of each horn. Regardless of the method used, it is important to expand the uterine lumen sufficiently to dislodge the embryos. Embryos are examined and held similarly to canine embryos at room temperature until transferred or cultured.

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Emerging roles for vitamin D and prolactin in canine male reproduction

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Abstract

Vitamin D and prolactin, known for their traditional roles in calcium homeostasis and lactation, respectively, are emerging as important factors in human prostatic health and semen quality. Recent research to elucidate the function of these elements in human male reproduction will be briefly reviewed with an emphasis on exploration of new avenues of study in reproduction in the stud dog.

Keywords: Vitamin D, prolactin, dog, reproduction

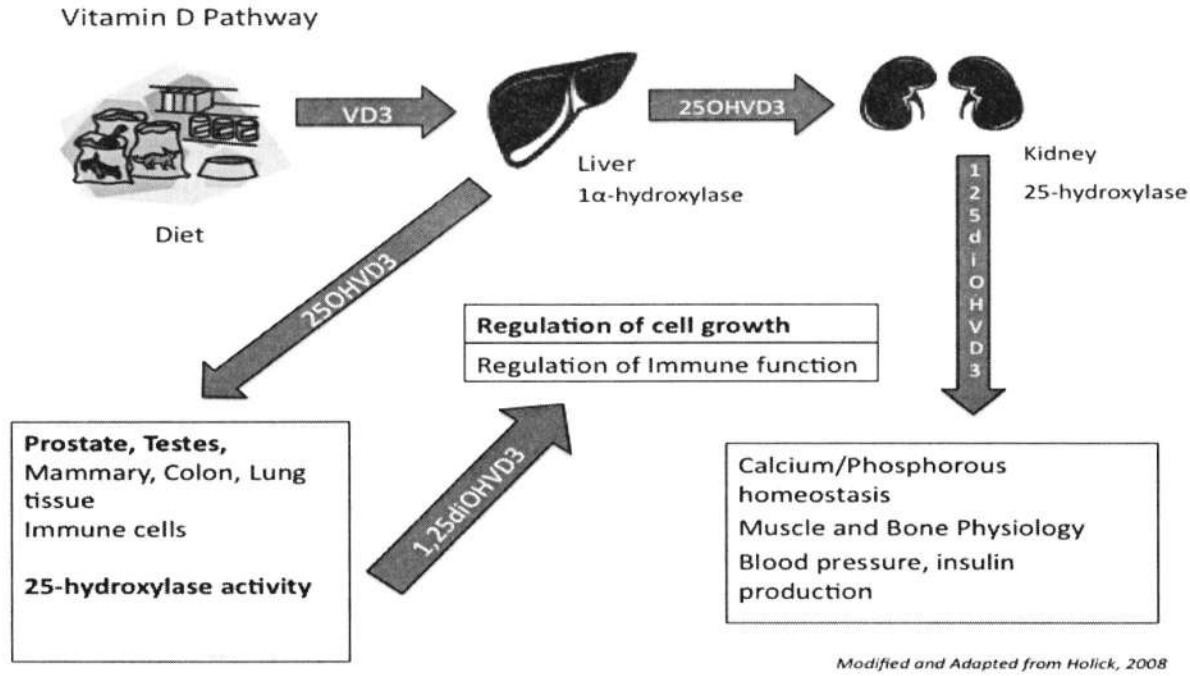
Introduction

From a veterinary clinical perspective, vitamin D (VD) and prolactin (PRL) are well known for their roles in preventing rickets and in the normal physiology of preparing the mammary gland for lactation and for maternal behaviour in the dam, respectively. However, it appears that multiple physiological and or pathological roles can be attributed to both these factors such as a decreased mortality risk with prostatic cancer[1] and benign prostatic hyperplasia[2] (BPH) in men with adequate VD intake and a role in decreasing semen quality and prostate size when PRL levels are greater than normal.[3] This brief review of the literature will look at the effects of both these compounds in male reproduction and possible new avenues of research into prostate health and semen quality in the dog.

The physiology of vitamin D

Vitamin D is a fat-soluble vitamin and pro-hormone that exists in two forms; cholecalciferol (VD2) from plant sources and ergocalciferol (VD3) found in animal sources.[4] Unlike humans, carnivores cannot synthesize VD3 through the action of ultraviolet (UV) radiation on the skin and as a consequence, it must be obtained strictly through the diet from sources such as liver or fish oils.[4] Once ingested, VD3 is stored or converted in the liver to 25 hydroxycholecalciferol (25OHVD3), the main circulating form in plasma. Enzymatic conversion to VD3 is dependent on the amount of VD3 ingested, liver function and circulating 25OHVD3 concentrations in plasma.[5] In calcium and phosphorous homeostasis, 25OHVD3 is further converted by 1α-hydroxylase in the kidney to the biologically active form 1,25 dihydroxycholecalciferol (1,25diOHVD3), otherwise known as calcitriol or 24,25 dihydroxycholecalciferol in the kidney. 1α-hydroxylase is controlled by a variety of factors such as the plasma concentrations of calcium, phosphorus, parathyroid hormone and VD3 metabolites.[5] However, it is the non-traditional role of VD3; mainly its anti-proliferative effects and its action as a mediator of cell differentiation and apoptosis in multiple tissues such as the prostate and testis that is of greatest interest.[6,7] The enzyme 1α-hydroxylase is found in multiple tissues, including the prostate gland in humans and rats.[8] After conversion to calcitriol, this biologically active form binds to the cytosolic vitamin D receptor (VDR), a heterodimer is formed with retinoid X receptor (RXR) in order to pass into the nucleus and bind to VDR response elements (VDRE) to initiate nuclear transcription events and/or to suppress them.[7] With regards to male reproduction the VDR has been identified in human and rat prostate and testes (seminiferous tubules and spermatozoa)[9-11] as well as in prostatic tissue in the dog (Figure).[12,13]

Figure



Vitamin D and the prostate

Recent cancer statistics in the human male population have shown that prostate cancer is the second most diagnosed cancer and has the second highest mortality rate in men next to lung cancer,[14] while BPH was found to occur in three out of four men in their seventh decade.[15] Although, the occurrence of prostatic carcinoma is relatively rare in the dog at less than 4% reported by Mukaratirwa,[16] BPH prevalence is similar to their human counter-parts with an estimated >80% of dogs having BPH by the age of four years.[17,18] Early epidemiological studies into risk factors of prostate cancer[19] found a strong negative association of UV exposure and mortality risk of prostatic carcinoma from which the authors hypothesized a role for VD in the pathogenesis of the disease. Conversely, meta-analysis of 11 relevant studies investigating risk of prostate cancer and VD levels however, showed no association between circulating 25OHVD levels and prostatic cancer risk.[20] However, the role of VD appears to be more complex than simply looking at circulating serum levels. Certain prostate cancer lines do not express the gene for 1α-hydroxylase and increase production of 24-hydroxylase to inactivate 25OHVD and early clinical trials in human medicine have shown a synergistic effect among calcitriol, dexamethasone and ketoconazole – a potent inhibitor of 24-hydroxylase – that resulted in a synergistic decrease of prostate specific antigen (PSA) levels in human patients[21] – a biological marker for prostate disease in men.

In the case of BPH, prostatic cells undergo hyperplasia – not dysplasia – and therefore retain the characteristics of normal prostatic cells. This process is mediated through the action of the androgen dihydrotestosterone (DHT), formed from testosterone (T) by the action of 5α-reductase within the prostatic epithelium.[17] Although greatest treatment effects are mediated through the action of 5α-reductase inhibitors such as finasteride, a multifactorial influence on prostate size exists, of which the VDR has attracted some attention. Both *in vitro* and *in vivo* studies in rats and humans[22-27] have tested VD analogues such as elocalcitol with affinity for the VDR, yet without the calcemic effects seen with the use of calcitriol. Indeed, it is the toxic effects of VD that limit the dose and frequency of calcitriol in treatment studies.[21] Elocalcitol, used on human BPH tissue culture *in vitro* was shown to decrease the number of cells via apoptosis in the presence of T, by more than 40%.[24] In the same study, a similar

effect was seen in decreasing rat prostate weight *in vivo* by 30% compared to 40% in the finasteride treated group.[24] These *in vivo* studies also showed no effect of elocalcitol on T and luteinizing hormone (LH) levels suggesting control of proliferation downstream of androgen action and lack of significant side effects on the pituitary and testes.[23] Adorini et al tested elocalcitol in six beagle dogs prior to clinical trial in humans and observed a decrease in prostate weight over a nine month treatment period that persisted until the end of the two month recovery period.[22] However, statistical significance could not be reached possibly due to the small sample size.

Vitamin D and semen quality

There are few studies in the literature involving VD and its role in male fertility, namely semen quantity and quality characteristics. In rats, it was shown that fertility rate decreased in vitamin D deficient male rats based on pregnancy rates and presence or absence of sperm in the vagina after copulation compared with the VD supplemented controls.[28] Furthermore, reduced testicular and epididymal sperm count, decreased numbers of Leydig cells, degenerative changes in germinal epithelium and lowered glutamyl transpeptidase activity in Sertoli cells was shown in a study by Sood et al and supported by identification of the VDR in rat Sertoli cells.[29-31] To determine the role of the VDR, Kinuta et al studied VDR null mice and found a dramatic reduction in sperm count and sperm motility compared with controls.[32] Histologically, thinning of the seminiferous epithelium was noted with dilated seminiferous tubules and decreased or infrequent spermatogenesis in the testes of the mice. Interestingly, those VDR null mice that had calcium supplementation with normal serum calcium levels did not show signs of decreased fertility.

In humans the VDR has been identified in the head of sperm cells but is lacking in the neck and tail region.[33] Cholesterol efflux – a priming event in the phosphorylation of proteins leading to human sperm capacitation – was increased in the presence of calcitriol. Also, an increase in phosphorylation of tyrosine and threonine suggests that the VDR has a role in capacitation and survival of sperm.[33] Blomberg-Jensen et al were able to identify the VDR and the enzymes of VD metabolism in the human testis, epididymis, prostate, and seminal vesicles in varying degrees.[11] Currently, canine studies investigating presence or action of VD or the VDR in testes or sperm do not exist.

The physiological role of prolactin

Prolactin is a 23kDa pituitary peptide hormone related to growth hormone (GH) and placental lactogen (PL) and is produced by the lactotroph cells of the anterior pituitary. It is well known for its role in lactogenesis in the female, however, it is also known to have a role in regulation of male reproduction and is involved in reproductive pathologies in men.[3, 34] It exerts its own negative feedback, through the cerebrospinal fluid, on the dopaminergic cells of the hypothalamus (HT).[35] The main effect of PRL is on the interstitial cells of Leydig by up-regulating LH receptors and increasing the affinity of the Leydig cells for LH thereby stimulating release of T.[35] Testosterone, in turn, stimulates Sertoli cells to produce estradiol-17-β (E_2) and androgen binding protein (ABP) and through negative feedback on the dopaminergic cells of the HT decreases PRL secretion.[35] The main hormone of up-regulation of PRL secretion is thought to be E_2 through a direct effect on lactotroph cells.[35] However, in cases of hyperprolactinemia in men, where greater than physiological levels of E_2 are present, it also exerts an inhibitory effect on gonadotropin releasing hormone (GnRH) release from the HT causing decreased secretion of LH and follicle stimulating hormone (FSH) from the pituitary, a response not seen in normal physiological conditions.

Prolactin reference ranges, ultradian and circannual patterns of PRL secretion have recently been published for the male dog,[36-39] although a difference in type of assay among these studies should be noted. The ranges published by Corrada et al, using homologous enzyme immunometric assay are summarized in the table. Of note, Beagle dogs had, on average, much higher PRL levels than crossbred dogs and German Shepherd dogs sampled in this study. This same group was able to show a circannual variation in PRL levels with an association of higher levels with increased daylight hours (November, December and January compared to May, June, July in the southern hemisphere). Although, Kreeger et al

recorded nadir during the fall months of October and November, taking latitude into account, both studies saw similar patterns with respect to hours of daylight.

Although, mean PRL concentration measured using radioimmunoassay (RIA) did not differ widely between breeds with normospermia; Urhausen et al found a significant difference between these values in Fox Terriers compared with Great Danes, the latter being lower. It was difficult to draw breed specific differences, however, possibly due to the close genetic relationship between the Fox Terriers enrolled in the study. A sharp increase in PRL levels was observed after thyroid stimulating hormone (TSH) injection in this same study. Mean values of PRL remained within the range specified by Corrada et al, yet it is important to note that sampling was only done once prior to TSH stimulation. Therefore normal PRL fluctuation was not accounted for. Although, semen quality parameters are affected in human males, the study by Koivisto et al, determined that semen parameters and libido remained unchanged after induced short-term hyperprolactinemia of three weeks duration.[40]

Table. Prolactin values in dogs

Range (Mean ± SE) (ng/ml)	0 - 6.0 (2.7 ± 0.2)
Mean Baseline ± SE	1.4 ± 0.6
Pulse frequency (peaks/6hr)	1-2
Pulse duration (mins) (Mean ± SE)	15-75 (45 ±11)
Pulse amplitude (ng/ml) (Mean ± SE)	1.7-2.4 (1.7 ±0.4)

Adapted from Corrada et al 2006

Prolactin and the prostate

Prolactin and its receptor (PRLR)[41,42] have been identified in human and rat prostatic epithelial tissue. Prolactin has been found to be a necessary component for prostatic epithelial growth and survival in culture.[34,43] Both *in vivo* and *in vitro* mouse studies showed PRL to affect growth and differentiation of the prostate[44,45] and sensitized the prostatic epithelial cells to androgen effects through synergism between T and PRL producing an increase in 5α-reductase activity.[46] This resulted in marked increases in the weight of the gland with histologically detectable hyperplastic changes[47] under conditions meant to mimic hyperprolatinemia. Prolactin and PRL binding sites were found intracellularly in canine prostatic cells,[48] however, the changes noted in the mouse and rat studies were not found in the dog and increases in cell proliferation were seen only in those cultures supplemented with bovine and dog serum alone and not with any steroid hormones or PRL.[49,50] The effect of PRL in the dog could be mediated through downregulation of DHT levels. Helmerich et al were able to show that pretreatment with PRL decreased prostatic tissue levels of DHT with subsequent increased T and was significantly different from controls.[51] In this same study, treatment with bromocriptine significantly increased prostatic DHT compared with controls.

A regulatory role of PRL was further supported by the study by Robertson et al. In PRLR knock-out mice, the ventral lobe of the prostate was 20% heavier compared to controls. However, the ratio of epithelial cells to stroma within the dorsal lobe was decreased in PRLR -/- mice. They found that castrated PRLR knock-out mice had greater reduction in prostate size than in normal controls suggesting that PRL and T act together on development of the ventral lobe, with a PRL dependent regulating effect under normal physiological conditions of the hormone.[52] This difference in size between PRL null and control mice disappeared at one year of age suggesting there is a transient affect of PRL during prostate development.

Conflicting findings on the role of PRL in male prostate physiology are apparent in the literature, however, it appears that this can be attributed to dose-dependent effects and chronicity as seen with other pituitary hormones such as GnRH agonists. The PRL feedback loop appears to take longer for effect when compared with other hormones.[53] Further research is needed; especially in the dog, as even less information is available in this species. This absence of information opens a new door to studying the prostate and conditions such as BPH.

Prolactin and semen quality

It is well known that conditions of hyperprolactinemia are associated with semen quality and decreased libido in both rodents and men and depending on the severity of the hyperprolactinemia and chronicity of the condition, hypogonadism may be present.[35,54-58] The main effects on semen quality include oligozoospermia, asthenozoospermia and teratozoospermia in these species. Many of these defects can be corrected with treatment of dopamine agonists or PRL antagonists although slight elevations in serum PRL seem to have little effect on male reproductive characteristics. In contrast to the female, PRL deficiency appears to have no effect on male fertility as is shown with PRL null mice and rats being able to sire normal litters with no reduction in libido or semen quality.[40] In the dog, Koivisto et al found a significant, if small, increase in mean straight-line velocity in the spermatozoa of those dogs treated with cabergoline.[40]

Conclusions

Vitamin D and prolactin have been shown to be involved in reproductive physiology. Ongoing research is helping to better understand these elements and to determine how their effects may be used to develop new treatment strategies for both prostatic and infertility issues. Although, species differences exist between human and canine reproduction, basic reproductive physiological characteristics may warrant further study to elucidate the possible roles of VD and prolactin in the stud dog and promises to be a fascinating area of research in this species.

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Treatment of prostatic disease

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Abstract

Prostatic disease is common in dogs, with the majority of intact dogs over the age of five years exhibiting benign prostatic hyperplasia, which then predisposes the dog to prostatitis, prostatic cysts, and prostatic abscessation. Prostatic neoplasia occurs in both neutered and intact male dogs. Clinical signs of prostatic disease can be quite severe and include pain, dysuria, dyschezia, and, in the case of neoplasia, death. Treatment options for prostatic diseases are the subject of much active research, since man is the only other known animal with significant prostatic disease. This review will discuss the current state of medical and surgical treatment for prostatic disease in dogs.

Keywords: Prostate, prostatitis, benign prostatic hyperplasia, prostatic neoplasia, canine

Introduction

Prostatic disease affects a significant proportion of dogs with reportedly over 80% of intact male dogs over five years of age[1] and over 95% of intact male dogs over nine years of age[2] exhibiting benign prostatic hyperplasia (BPH), which then further predisposes them to developing prostatitis, prostatic abscesses, and prostatic cysts. Prostatic neoplasia affects both intact and neutered males, with the latter group showing an increased risk for prostatic neoplasia.[3-5] Other reviews have covered the pathogenesis and diagnosis of canine prostatic disease.[1,6,7] This review will focus on current treatment options.

Prostatic anatomy and physiology

The prostate in the dog is a bilobed, oval to spherical-shaped organ with both a dorsal and ventral sulcus located in the cranial pelvic canal or in the caudal abdomen. The proximal urethra runs through the prostate between the two lobes. Testosterone is converted to dihydrotestosterone (DHT) via the enzyme 5α-reductase and it is this androgen, DHT, which stimulates prostatic development, growth, and secretions.[8] The enzyme 5α-reductase is found in two isoenzymes in the body, type 1 and type 2. Each isoenzyme is encoded by a different chromosome, but common coding sequences indicate a common evolutionary precursor. Isoenzyme type 1 is found throughout the body, including the skin, liver, and prostate. Isoenzyme type 2 is found predominantly in the prostate and other genital tissue. Testosterone and DHT both bind to the same androgen receptors and cause the same effects. The binding of DHT to the androgen receptor, however, is much tighter and of longer duration than that of testosterone. The resultant effect is that lower concentrations of DHT cause an amplified response compared to testosterone.[9,10]

Benign prostatic hyperplasia

The majority of intact male dogs will develop BPH by the age of five years. The disorder consists of both cellular hyperplasia and hypertrophy.[11] Most dogs with BPH do not show any clinical signs, and therefore require no treatment. Clinical signs may include sanguinous prostatic fluid dripping from the prepuce, hemospermia, hematuria, dysuria, constipation, or tenesmus.[12] The prostate may be palpated per rectum and would feel bisymmetrically enlarged and non-painful. Ultrasound evaluation of the prostate allows accurate measurement of the prostatic lobes and visualization of the parenchyma. Prostatic parenchyma in a dog with BPH should be uniformly echogenic. Evaluation of the third fraction of the ejaculate often reveals a marked number of red blood cells. Treatment should be considered in any dog displaying clinical signs to resolve both discomfort from constipation or dysuria and potential subfertility from hemospermia.

BPH treatment

Resolution of prostatic hyperplasia is achieved by removing the androgen source of prostatic stimulation. In a dog without a breeding career, the treatment of choice is bilateral orchidectomy. Castration removes the source of testosterone and consequently eliminates the production of DHT. In the absence of DHT stimulation the prostate rapidly reduces in size 80% by 12 weeks after castration.[13] Castration will also eliminate the risks of testicular tumors and future unwanted litters of puppies.

In dogs considered valuable breeders, castration is obviously not indicated. In these cases, medical treatments aim at disrupting the androgen stimulation on the prostate. This review will consider treatments including 5α-reductase inhibitors, androgen receptor inhibitors, androgen antagonists, aromatase inhibitors and other antiestrogen therapies, and gonadotropin releasing hormone (GnRH) agonists. Treatments of the past that are no longer in widespread use due to serious adverse effects, such as estrogens, will not be discussed in this review. In men, lower urinary tract symptoms are common sequelae of BPH and an effective treatment is the use of α_1-adrenergic receptor antagonists to relax smooth muscle in the lower urinary tract.[14] As this is not usually a concern in dogs, this treatment is not used and will not be discussed.

5α-reductase inhibitors. In the United States, the most common medical treatment for BPH is the daily administration of finasteride, an azasteroid which at human clinical doses selectively inhibits the action of 5α-reductase type 2, thus reducing the conversion of testosterone to DHT.[9] Because testosterone itself is not inhibited, its effects on libido and spermatogenesis are preserved. The removal of DHT, the active androgen in the prostate, significantly decreases androgenic stimulation to the prostate, which consequently reduces in size due to apoptosis of prostatic cells.[15]

Dose ranges have not been definitively established for the use of finasteride in the dog, though the safety margin appears to be quite large. Finasteride in its popular commercial forms marketed for men comes in 5 mg tablets (Proscar™, Merck, West Point, PA) and in one mg tablets (Propecia™, Merck, West Point, PA). Perhaps because the former is marketed for treatment of BPH in men and the latter for treatment of male pattern baldness, veterinarians have used the five mg tablet (Proscar™) as a once-daily treatment for dogs with BPH. At a dosages ranging from five mg/dog once daily (0.5 mg/kg in a 10 kg beagle dog) up to 45 mg/kg once daily, a 50-70% reduction in prostatic volume has been noted after 16-53 weeks of treatment.[16-20] This ends up being a much higher dose, pound for pound, in dogs than men. The dose in men is 5 mg/person once daily, which for a 200 lb man works out to be about 0.05 mg/kg daily. At this dose, prostatic volume in men was reported to decrease by 18% after one year of treatment, after which further reductions in size were insignificant, compared to a 14% increase in volume in the placebo group.[21] A study reducing the dose to 0.1-0.5 mg/kg in dogs achieved a 43% reduction in prostatic volume after 16 weeks of daily treatment with no negative effects on serum testosterone concentrations or semen quality.[22]

In humans, side effects include erectile or ejaculatory dysfunction and teratogenic effects in pregnant women, predictably with regard to sexual differentiation in male fetuses. Problems with libido and fertility have not been reported with dogs, in fact reports indicate normal fertility and libido in dogs on finasteride.[19] Teratogenic effects should not be an issue in dogs since female dogs do not receive finasteride. Some veterinary clinicians have reported concerns about male dogs passing the drug on to females through the semen in mating, but passage of the drug in the semen has not been shown to be a concern in humans (who have sexual relations during pregnancy, whereas dogs do not) and the half-life of finasteride is short enough to not be a teratogenic concern even if it were passed in the semen.

Another concern is the use of finasteride in dogs used in natural mating programs. Semen in natural mating in canines is deposited in the cranial vagina and then it is thought that the continual secretion of large volumes of prostatic fluid during ejaculation of the third fraction pushes the sperm-rich fraction through the cervix into the uterus. Use of finasteride greatly decreases the volume of the third fraction, raising the concern that fertility will be decreased in natural matings using male dogs taking finasteride. This concern has lead to a variety of dosing strategies among clinicians ranging from discontinuing use of finasteride a week or two before an anticipated mating, reducing the dosing schedule

to once every few days, or only putting the male on finasteride once a year for a few months, as examples. Each of these strategies is untested and purely anecdotal. One study has tested the fertility of dogs on an active finasteride protocol and found no affect on fertility,[19] so it appears that while the concern regarding use of finasteride during natural mating programs makes sense, it may be unwarranted. Certainly more studies on appropriate dosing regimens for finasteride are needed in the dog. The conventional wisdom at this point would be to continue finasteride treatment (somewhat) continuously until the breeding career of the dog is over, and then consider castration.

Antiandrogen therapy. Osaterone acetate (Ypozane®, Virbac, Carros, France) is a testosterone analogue with potent antiandrogenic activity attributed to competitive binding to androgen receptors, as well as the overall reduction of androgen receptors, reduction of 5α-reductase, and the inhibition of testosterone transport into prostate cells.[23] In one trial 0.25 mg/kg was administered orally once daily for seven days to 73 dogs with clinical signs of BPH. By 14 days after the start of the trial, nearly half of the dogs had resolution of clinical signs and an average reduction in prostate volume of 38% was noted. By six months after the start of the trial, 84% of dogs had resolution of clinical signs.[23] Using this same dosing regimen, it was determined that peak serum concentrations were reached by day seven, which may explain the initial rapid effect and then slow tapering of effects in the following weeks.[24] Semen quality and fertility do not seem to be negatively affected by osaterone and may, in some cases, improve.[25] Ypozane® is marketed in France and available in some countries in the European Union. It is not licensed in the US.

Progestins exhibit antiandrogen activity and therefore have been used to treat BPH. The antiandrogenic action of progestins is likely due to competitive binding with the androgen receptors and/or suppression of luteinizing hormone (LH) secretion via negative feedback.[26] Dogs in one study were treated for BPH with medroxyprogesterone acetate and, while 84% showed a reduction in clinical signs, only 53% showed a reduction in prostate volume after six weeks of treatment.[27] No effect was noted on semen quality or libido. Concerns regarding the development of diabetes mellitus or mammary nodules has precluded its popular use for BPH treatment.

Delmadinone acetate (Tardak®, Pfizer Animal Health, Sandwich, Kent, UK) is a progestin that is 17 times more potent in antiandrogenic activity than progesterone.[23] Treatment of 69 dogs with clinical signs of BPH using a single intramuscular or subcutaneous injection at 3 mg/kg of delmadinone resulted in complete remission of clinical signs by 14 days after the injection in nearly half of the dogs and in 83% of the dogs by six months after the injection. At 14 days after the injection, a 28% reduction in prostate volume was noted.[23] One of the 69 dogs in the trial developed hypoadrenocorticism, which required treatment to resolve. In another study 1.5 mg/kg delmadinone was administered as a subcutaneous injection at 0, one, and four weeks of the trial and ACTH and cortisol levels were monitored during the trial. Adrenocorticotropic hormone stimulation tests were also conducted. The study noted a significant decrease in basal and two h post-ACTH stimulation concentrations of cortisol in treated dogs compared to control dogs. The authors concluded that treated dogs may be at risk for developing glucocorticoid insufficiency during treatment if subjected to stressful events.[28] Other side effects were of minimal importance, transient, and affected very few of the dogs; these included increases in appetite, behavior changes, vomiting, diarrhea, asthenia, polyuria, and polydipsia. In a separate study, male beagle dogs given an single injection of 1 mg/kg of delmadinone showed a temporary change in the maturation of epididymal sperm cells.[20] Tardak® (Pfizer Limited, Sandwich, UK) is marketed in the UK and currently licensed in Austria, Belgium, Finland, France, Luxemburg, Netherlands and the UK. It is not licensed in the US.

Antiestrogen therapy. Estrogens have been thought to play either a causative or permissive role in the pathogenesis of BPH.[29] Use of estrogens as a treatment for BPH is no longer in favor due to potentially severe side effects including pancytopenia. Use of antiestrogen therapies, such as estrogen receptor antagonists and aromatase inhibitors have been used to relieve clinical signs of BPH and reduce prostate volume.

Tamoxifen citrate is an antiestrogen drug that has been given at a dose of 2.5 mg/dog once daily for 28 days in male dogs with clinical BPH. Researchers measured testicular parameters, semen parameters, libido, prostatic volume, and serum testosterone.[30,31] Prostatic volume decreased by 28-50% during the treatment period, but returned rapidly to at or below pretreatment size after treatment ceased. All other parameters measured showed dramatic decreases during treatment, some disappearing altogether, but all gradually returned to pretreatment levels by the end of the monitoring period. Volume of the third fraction of the ejaculate decreased to a few drops or was absent. Maximum scrotal width decreased and the testes softened. Libido decreased and became nonexistent. The volume of the sperm-rich fraction of the ejaculate decreased to nearly nonexistent levels with a corresponding decrease in total sperm numbers. Sperm motility, normal sperm morphology, and serum testosterone concentrations all decreased during the treatment period. After parameters returned to normal limits, three of the male dogs were bred to females which conceived and whelped normal litters. No systemic side effects were noted during the treatment period. Tamoxifen citrate, marketed under various trade names by different pharmaceutical companies, is primarily used as a treatment for breast cancer in women. Tamoxifen may represent a treatment option in very select cases. It offers a rapid prostatic response and an apparently reversible contraceptive effect, at least with limited use.

Anastrazole (Arimidex®, AstraZeneca,London, UK) is a potent, highly selective aromatase inhibitor with no intrinsic hormonal activity that has replaced tamoxifen in many breast cancer treatment protocols for women.[32] Given to dogs at a dose of 0.025 mg/dog once daily for 28 days, a rapid reduction in prostate volume of 21% was noted with no significant changes in libido, testicular consistency and scrotal diameter, or sperm volume, count, motility, and morphological abnormalities.[31] No hematological or other clinical abnormalities were noted. Anastrazole may present veterinary practitioners with a more rapid alternative to protocols using finasteride.

GnRH agonists. Deslorelin acetate (Suprelorin®, Virbac, Carros, France) and azagly-nafarelin (Gonazon®, Intervet, Angers Technopole, France) are potent GnRH agonists that shut down LH release by desensitizing the pituitary gonadotrophs to GnRH and the Leydig cells to LH.[33] Gonadotropin releasing hormone agonists have been used in domestic dogs and in wild carnivores as reversible contraceptives.[34-38] Spermatogenesis and libido are suspended after an initial stimulatory period and the prostate gland and testes decrease in volume up to 55%.[39] These parameters remain suppressed throughout the duration of the treatment, slowly returning to normal ranges usually two to three months after cessation of the treatment.[34,38-42] Male fertility appears to be unaffected after recovery from the treatment.[39,40] Agonists of GnRH may be very useful in treating dogs for BPH in situations when non-surgical, reversible contraception is also a goal during the treatment period. Gonadotropin releasing hormone implants are not available commercially in the US at the present time.

Prostatitis

Dogs experiencing BPH are predisposed to developing prostatitis. Reports of prostatitis in castrated male dogs are rare and affected dogs often have a history of recent castration prior to presentation. Clinical signs of prostatitis will vary largely depending on the chronicity of the infection, with acute cases showing more serious, painful clinical signs and chronic cases often presenting as subclinical. Clinical signs relate to pain, but may manifest as back pain, abdominal pain, a painful stiff gait or depression. Semen quality and libido may be diminished. Hemospermia, hematuria, pyospermia, and fever may be present. Because prostatic fluid constantly flows both retrograde into the bladder as well as antegrade out the prepuce, these cases can be misdiagnosed as urinary tract infections. Per rectal palpation of the prostate will likely elicit pain in acute cases, but may not in chronic cases. The prostate will feel enlarged in acute cases and may not be bilaterally symmetrical, especially if abscessation is present. Some chronic cases of prostatitis may not have an obvious enlargement as fibrosis may have reduced the size of the prostate. Evaluation of the third fraction of the ejaculate is very helpful, if the dog is not too painful to cooperate with manual collection. Collection of a sample via fine needle aspiration is discouraged because of the concern for seeding the needle track with the infectious agent. The prostatic

fluid will often have a marked number of neutrophils that may show degenerative changes and may have intracellular bacteria. Lack of neutrophils in the third fraction does not entirely rule out prostatitis, as neutrophils may be in a distinct segment of the prostate, not communicating with the secretory ducts. Culture of the third fraction to determine the causative agent should be performed if prostatitis is suspected. While *Escherichia coli* is the most common pathogen in canine prostatitis, any opportunistic bacteria ascending from the urethra may cause the infection. Fungal causes are possible, but much less common and usually part of a systemic fungal infection.[1,43] A complete blood count will often reveal a regenerative leukocytosis, but some dogs may be leukopenic. Ultrasound evaluation of the prostate is valuable and will usually show a heterogenous echogenic appearance to the prostatic parenchyma, with or without larger hypoechoic regions corresponding to abscessation.

Prostatitis treatment

Because BPH predisposes dogs to prostatic infections, treatment aimed at reduction of the hyperplasia is warranted. For dogs without valuable breeding potential and no signs of systemic infection, castration coupled with antibiotic therapy is the preferred treatment. Otherwise, choosing from one of the medical options discussed above in this review for BPH treatment should accompany treatments directed at the infection. Antibiotic therapy should be based on culture and sensitivity results and on consideration of the unique physiology of the prostate. Due to the profound inflammation present in acute prostatitis, the blood-prostate barrier is less functional and allows adequate diffusion of drugs that otherwise would not reach therapeutic concentrations in the prostate. Drugs such as broad-spectrum penicillin derivatives or a third-generation cephalosporin may initially be used to good effect. Once the blood-prostate barrier heals after initial improvement, however, diffusion across the barrier is limited to drugs containing specific pharmacokinetic properties and the antibiotic choice must be switched to an antibiotic with those properties. Drug penetration occurs via passive mechanisms of concentration gradients and diffusion. The blood-prostate barrier permits access only to lipophilic drugs and those not highly bound to proteins. In addition, the pH of the prostate is more acidic than the blood (canine prostatic pH ranges from 6.1 to 6.5).[44,45] The phenomenon of ion trapping further determines the concentrations of drugs across the membrane. Each drug will have a charged fraction (ionized) and an uncharged fraction. The uncharged fraction of a lipophilic drug, in a stable system, will equilibrate on both sides of the membrane. The charged portion of the drug, however, will concentrate more on one side or the other, depending on the differing pH on each side. The drug will be most concentrated on the side with the greatest ionization (the greatest charge).[46] Weak bases will therefore concentrate in the acidic canine prostatic fluid.

Antibiotic drugs that have proven efficacy in treating prostatic infections are discussed below. Whichever antibiotic is chosen, treatment should continue for between four to six weeks in acute cases and six to eight weeks in chronic cases. The dog should be re-examined after the end of the treatment to confirm resolution of the infection. Treatment for the standard 10-14 days will usually result in recurrence of the condition shortly after cessation of antibiotic treatment and will predispose the dog to resistant infections.

Trimethoprim. Trimethoprim has the necessary properties to allow diffusion across the blood-prostate barrier and is a weak base with a pK_a of 7.4, therefore concentrating well in the acidic environment of the canine prostate.[47] Trimethoprim has good broad-spectrum activity, but is not effective against anaerobic infections.

Fluoroquinolones. The fluoroquinolones are amphoteric or zwitterionic in that they are neither purely acidic nor basic, but have qualities of both in clinical settings. They essentially have two ionizing groups, one positively charged and one negatively charged. At a pH somewhere in between the two groups, there is a minimal amount of charged drug. This is the isoelectric point. At pH values higher or lower than the isoelectric point, the amount of charged drug increases. So, if an amphoteric drug has an isoelectric point close to the pH of plasma, the drug will tend to concentrate in areas where the pH is higher or lower than that of plasma. This is the case with fluoroquinolones and why their concentrations

are higher in the prostatic environment.[46] The fluoroquinolones have a good broad-spectrum of activity and enrofloxacin is effective against mycoplasma infections. Fluoroquinolones do not act efficiently against anaerobic infections.

Macrolides. The macrolides diffuse very well into the prostate, but have poor action against gram-negative bacteria.[48] They should not be used until a sensitivity analysis has been obtained to show that the pathogenic bacteria are gram-positive organisms sensitive to the drug. Examples for veterinary use include erythromycin and tylosin.

Chloramphenicol. Chloramphenicol attains good concentrations in the prostate and exhibits good activity against many anaerobes. The toxicity of chloramphenicol in humans is most likely not a concern in adult male dogs. Chloramphenicol, therefore, may be a good choice for an anaerobic prostatic infection.

Prostatic abscessation

Dogs with prostatic abscesses should be treated with the same protocols as dogs with prostatitis, using treatment targeted at BPH and appropriate antibiotics for the infection. In addition, active drainage of the abscess is often necessary either via surgical procedures or percutaneous, ultrasound-guided drainage. The latter procedure remains controversial because of the concern for seeding the needle track with infectious bacteria.

Surgical drainage may be accomplished by marsupialization, Penrose drainage, or omentalization. Detailed description of each of these techniques is beyond the scope of this paper, but has been recently reviewed[49] and is covered in veterinary surgical texts. A summary of each technique will follow.

Prostatic abscessation treatment

Prostatic omentalization. Omentalizaiton is currently the procedure of choice for surgical drainage of prostatic abscesses.[49] The omentum provides an alternate vascular and lymphatic supply and functions well in the presence of infection. As such, it has been used in multiple small animal surgical procedures.[50] The procedure of placing the omentum through the capsule of 20 dogs with prostatic abscessation (intracapsular omentalization) resulted in complete resolution in 19 dogs, with one dog showing recurrent abscessation and requiring Penrose drain placement.[51] Minimal to no post-operative complications were reported and most dogs were discharged to go home with their owners 48 hours after surgery. If the abscess is in a paraprostatic retention cyst, and not intracapsular, omentalization may still be used to good effect.[52]

Penrose drainage. Penrose drain placement was the treatment of choice before the advent of omentalization techniques. Placement of a Penrose drain within the abscess and leading out through the abdominal wall allows continuous drainage of the abscess. Various techniques are described that differ in the exact placement of the Penrose drains.[53] The time during which the drains are left in place varies with the technique from a few days to a few weeks. Active post-operative monitoring and care are necessary until drainage resolves, the drains are removed, and the wounds close. Complications may include recurrent abscessation, urinary incontinence, subcutaneous edema, anemia, sepsis, shock, hypokalemia, and hypoproteinemia.[54-57]

Marsupialization. Marsupialization is not commonly performed due to better post-operative results achieved by omentalization techniques. Marsupialization involves opening the abscess and suturing the edges to prevent the abscess from closing, allowing continual drainage. For treatment of prostatic abscessation, the edges of the opened abscess may be sutured to the external abdominal skin adjacent to the prepuce, or ventral or lateral to the anus.[53] The abscess is thus allowed continual drainage as long as necessary and antibiotic or antiseptic treatments may be placed directly into the abscess. Drainage reportedly usually continues for one to two months, but may continue for many months in

complicated cases. Active post-operative monitoring and care are necessary until drainage resolves, the drains are removed, and the wounds close. Complications potentially are the same as for Penrose drainage, and may also include fistula formation.[54-56]

Prostatic cysts

Prostatic cysts may be located within the prostatic parenchyma (retention cysts) or in a paraprostatic position. Prostatic cysts may not cause any clinical signs, or may result in urinary or defecation difficulties. Prostatic cysts also predispose the dog to developing abscessation and therefore removal is often recommended, even in the absence of clinical signs. Removal of retention cysts may be done using the techniques described for abscessation, preferably omentalization. Paraprostatic cysts or abscesses do not communicate directly with the prostatic parenchyma, and therefore local resection is often the treatment of choice.[53] Omentalization may also a good alternative.[52]

Prostatic neoplasia

While reported very rarely in a few other species, dogs are the only animal, besides man, with a known, significant occurrence of prostatic neoplasia. Adenocarcinomas or transitional cell carcinomas are the most common canine prostatic neoplastic diseases. While there are apparent similarities in the disease between the dogs and men, important differences also exist. As a result, many screening and treatment modalities used successfully in human medicine fail to be applicable in veterinary medicine. Prostatic neoplasia in men is often diagnosed in the early stages, thanks to heightened awareness and effective diagnostic screening tests (e.g., the prostate specific antigen [PSA] test), and is dependent upon androgens as growth factors. Androgen-deprivation is a foundation therapy for men with prostate cancer and they usually respond rapidly and favorably. Most prostatic neoplasia in men is benign or slow growing. In dogs, however, prostatic neoplasia tends to be highly aggressive and metastatic. Canine prostatic neoplasia is not androgen-dependent and is more commonly diagnosed in castrated males than intact males.[3-5] Reasons for the increased incidence of prostatic neoplasia in castrated dogs are unknown, but hypotheses include a loss of protective effects of androgens, a shift in the prostatic stroma from actin-positive smooth muscle cells to vimentin-positive mesenchymal cells, which may favor tumor formation, and increased longevity of castrated animals, predisposing them to age-related neoplastic diseases.[7,58] Clinical signs of prostatic neoplasia in dogs resemble those of other prostatic diseases including dysuria, dyschezia, and pain associated with the gait, back, or abdomen. Diagnosis is by history, clinical signs, an irregular, painful prostate on transrectal palpation, heterogenous echogenicity on ultrasound evaluation, neoplastic cells found on cytology, or biopsy results. Usually diagnosis is made at very late stages of the disease and survival times range from days to weeks after diagnosis.[7,59,60] Because of these differences, and the fact that no known treatment for canine prostatic neoplasia affects survival time, treatments are palliative and in some cases will result in an increased quality of life for a short time. Many treatment modalities, however, have potential, serious side effects that may result in the death or euthanasia of the dog. This underscores the need to tailor each recommendation to each specific clinical scenario. Clients should be made aware of potential complications, that treatments will not likely extend the life of the dog, but may alleviate clinical signs.

Prostatic neoplasia treatment

Surgery. Prostatic tumors tend to be highly aggressive and metastatic. If, however, there are no signs of metastasis, total prostatectomy may be a suggested therapy. There are some considerations, however, that make total prostatectomy in the dog less likely to produce an acceptable outcome. Even if metastasis has not been documented, there is a highly likelihood that it has happened nonetheless and will manifest itself shortly. Due to the location of the urethra inside the prostate, urinary incontinence and other signs of morbidity are common post-operative complications with total prostatectomy. Surgery has also not been shown to increase survival in many cases.[54,55]

Sub-total intra-capsular prostatectomy has also been tested, both using traditional surgical instruments and an Nd:YAG laser. In general, survival may be up to five times longer than after total

prostatectomy and with a lower incidence of urinary incontinence.[61-64] Survival times and post-operative complications are comparable to treatment with piroxicam alone (discussed below).[60]

Transurethral resection of the prostate has been reported in three dogs. Palliation of clinical signs post-operatively was rapid, but survival times remained short and complications included urinary tract infection, seeding of the tumor, and urethral perforation.[65]

Surgical therapy may alleviate clinical signs in some cases, but is associated with increased risk for complications and not associated with increased survival times. The decision to use surgery should be based on individual cases and rely on the skill and experience of the surgeon. Post-operative follow-up with systemic therapies to slow the spread of disease will likely aid in a more positive outcome.

Radiation. Radiation therapy has been tried in dogs without extending or improving the quality of life and resulting in some cases in severe adverse affects, including chronic colitis, gastrointestinal stricture or perforation, necrotic drainage and ulceration of the skin and subcutaneous tissues, osteopenia, urinary bladder thickening, chronic cystitis, urethral stricture, ileosacral osteosarcoma, pelvic limb edema, and perianal pain.[7,66,67] Survival time was not affected by the adverse effects, but quality of life decreased and owner expense increased. Radiation may play a role in future treatment regimens, but more work must be done to determine the best protocols.

Chemotherapy. The benefits of traditional chemotherapeutic agents have not been well documented with canine prostatic neoplasia. Work has been done to investigate the chemotherapeutic properties of some nonsteroidal anti-inflammatory drugs (NSAIDs). It is thought that cyclooxygenase (COX)-2 inhibition plays a key role through inhibition of angiogenesis, stimulation of apoptosis, and altering immune function.[68] One study evaluating the use of NSAIDs in the treatment of canine prostatic neoplasia found that a majority of normal and neoplastic prostatic cells expressed COX-1 and that only neoplastic cells expressed COX-2. The study also retrospectively evaluated dogs with prostatic neoplasia that were treated with NSAIDs and those that were not and found that survival time was significantly different between the two groups, with 6.9 months in the former group and 0.7 months in the latter.[60] The two NSAIDs evaluated were piroxicam and carprofen.

Bisphosphates are osteoclast inhibitors used in human medicine for treatment of skeletal metastasis of prostatic carcinoma. They have been tested in dogs and appear to have similar benefits of increasing bone density and decreasing pain in some patients.[69] Inhibiting osteoclast activity strengthens bone, which reduces pain and the risk of fracture. It also controls the humoral hypercalcemia of malignancy. Other benefits of bisphophates in cancer treatment include inhibition of cancer cell proliferation, induction of apoptosis of cancer cells, angiogenesis inhibition, matrix metalloproteinase inhibition, and cytokine expression alteration.[70]

Samarium-153-ethylenediamine-tetramethylene-phosphonic acid (^{153}SM-EDTMP), an injectable radiopharmaceutical, palliates and may have some curative properties in some restricted cases of canine skeletal metastatic disease (tumors less than two cm in diameter, not invading cortical bone, tumors in the axial skeleton, mineralized tumors, and those with high uptake of ^{99m}Tc-MDP during scintigraphy).[71] The drug is not currently easily accessible.

Dysuria therapy. As dysuria is a common effect of prostatic neoplasia, treatment may be focused on relieving this clinical sign. Tube cystotomy may be used, but owners should be aware of complications including urinary tract infection and dissemination of the tumor.[72] Presence of the tumor may also cause incontinence to persist. Placement of a metallic urinary stent has been reported, which resulted in immediate restoration of urinary function. The treatment is costly and complications may include loss of the stent, reobstruction, and incontinence. Seven of 12 dogs were scored as having an excellent outcome and mean survival time for all dogs was 20 days.[73]

Prostatic neoplasia treatment summary

No standard protocol for treatment of prostatic neoplasia in dogs exists, nor is it likely that a standard protocol ever will exist as long as most diagnoses are in the late, terminal stages of the disease, as individual patient variation and client wishes will always play an important role in deciding the correct treatment regimen. At the current time, there do not seem to be any treatments that reliably extend both life expectancy and improve quality of life, with the possible exception of COX-2 inhibitors. Some treatments do seem to offer palliative measures to decrease pain and other clinical signs and should be considered as available options on a case-by-case basis, considering owner expectations and concerns, and the current quality of life of the patient.

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Nutraceuticals—Pandora's box?

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The term nutraceutical is not a legal term, but was coined in the 1980's by a physician regarding oral compounds that were neither a nutrient or a pharmaceutical. The now defunct North American Veterinary Nutraceutical Council defined veterinary nutraceuticals as a non-drug substance in a purified or extracted form that is administered orally to provide agents for normal body function. Administering these products is done with the intention of improving the health and well-being of animals.[1] Nutraceuticals are classified as dietary supplements and fall under the regulation of the Food and Drug Administration (FDA).

The Dietary Supplement Health and Education Act of 1994 mandated that the FDA regulate dietary supplements as food and not as a drug. A dietary supplement is a product taken by mouth that contains an ingredient intended to supplement the diet. As a dietary supplement, nutraceuticals include a wide variety of products including: vitamins, minerals, herbs or other botanicals, amino acids, enzymes, metabolites and organ or glandular tissues. Nutraceuticals can be produced as tablets, capsules, liquids, soft gels, gelcaps, or powders.[2]

There are three categories of labeling claims: health claims, nutrient claims and structure/function claims. The manufacturer of the nutraceutical(s), the FDA or the Federal Trade Commission (in cases of advertising) are responsible for validating label claims. Manufacturers are allowed to make specific claims regarding health benefit(s) of their products, as long as there is adequate evidence to substantiate that those claims are not false or misleading. The label must include the following: a descriptive name of the product stating it is a supplement; the name and place of business of the manufacturer or distributor; a complete list of ingredients; and the net content of the product. The label must also include the following statement: "These statements have not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure or prevent any disease." There are no rules regarding the amount of the nutrient in a serving size of a nutraceutical. In addition, manufacturers and distributors do not need FDA approval to sell nutraceuticals.[3]

Products classified as a drug must be proven safe and effective before they are marketed to the public. Manufacturers of nutraceuticals and other dietary supplements are not required to prove safety or effectiveness of these products before marketing. The FDA is required to prove a dietary supplement is unsafe after it has been marketed before it will take action to limit or remove the product from the market. Unsafe nutraceuticals are brought to the FDA's attention through the use of Adverse Events Reporting. Reportable events are classified as either 'adverse' - any health-related event associated with the use of a dietary supplement that is adverse - or 'serious adverse event' - any event associated with the supplement that results in death, a life-threatening experience, hospitalization, significant disability or incapacitation, congenital anomaly or birth defect or requires medical or surgical intervention to prevent one of these stated outcomes.[4]

The perception of the public, however, is to view these products as entirely beneficial to health and in no way harmful to themselves or their animals. A tour of the World Wide Web will reinforce this idea. Phrases like: virtually no side effects; works with any other medication; safe; natural alternative; natural food based supplement; trusted by pet owners; empowered by nutraceuticals; natural benefits; pure and natural; improve health without giving actual medicines - lead the general public to believe there are no risks and all gains with the use of nutraceuticals.

The AVMA guidelines for complementary and alternative veterinary medicine (CAVM) include the use of veterinary nutraceutical therapy. This guideline states that although the quality of studies and reports pertaining to CAVM vary, it is the responsibility of the veterinarian to critically evaluate the literature and other sources of information regarding the use of these modalities. Veterinary recommendations for treatment with CAVM should only take place after a diagnosis is established. This diagnosis must be based on sound, accepted principles of veterinary medicine. The AVMA guidelines also state that recommendations based on CAVM modalities must be safe and effective and should be

based on available scientific knowledge and the medical judgment of the veterinarian. However, the guidelines do note that CAVM practices may differ from current scientific knowledge and what is routinely taught in veterinary curriculum.[5]

Results of experiments using nutraceuticals are difficult to interpret due to small animal numbers, low repeatability and response to these compounds varies between individuals. In addition to individual variability, one must also take into account the age (immature vs. mature), the breed, the selection pressure placed on different breeds/species (performance animals vs. food production animals), the environment (temperate vs. tropical) and overall ration (ration storage, amount fed to each individual, macro and micro nutrient quality and ratio) when evaluating the effectiveness of a particular nutraceutical.[6]

The lack of clear regulatory definitions in regards to the concentration of active ingredients and the presence of glycosidic and salt forms of these components in nutraceuticals allow for wide variability in the quality of the raw materials used to manufacture these products. Evaluations of 70 formulations of 25 different nutraceuticals revealed no nutraceutical showed consistently high quality. To the contrary, a number demonstrated consistently low quality. This wide variability in constituent quality was also noted when using whole food sources. These factors indicate that much closer regulation of the manufacture of nutraceuticals is required.[7]

In summary, the use of nutraceuticals offers client animals an opportunity to achieve optimal health if given in the correct formulation and for precise circumstances. The difficulties in identifying what nutraceutical or combination of nutraceuticals would benefit an individual animal with a particular malady is fraught with minimal regulatory oversight of these products, limited scientific data, misrepresentation of information to the general public, and wide variability in the quality of these bioactive compounds.

Some questions to ponder

- One of Webster's definitions of Pandora's box is a complex situation fraught with problems and pitfalls. How can DVMs guide clients in the use of nutraceuticals given the fact that more and more people are ordering products from the internet?
- How can one identify the best brand of nutraceutical for an individual animal's treatment?
- How does one evaluate the response to a nutraceutical?
- How long should an animal remain on a nutraceutical?
- At what stage of production (growth, breeding, pregnancy, lactation) should a nutraceutical be used or not used?
- How does one identify nutraceuticals that contain high bioavailable ingredients?
- How does one know if the quality of a particular nutraceutical is consistent from one lot to the next?
- What type of experimental design(s) would allow better evaluation of a nutraceutical for a particular syndrome like testicular degeneration?

Keywords: Nutraceutical, complementary and alternative veterinary medicine

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The use of protective AI cover sheaths improved fertility in lactating dairy cows

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Abstract

The objectives of this study were to evaluate the effectiveness of using a disposable sheath protector (SP) on top of the regular artificial insemination (AI) sheath to minimize contamination of the AI gun and to assess pregnancies per AI (PAI) in lactating dairy cattle inseminated with or without the use of SP. Services (n = 2843) during spring (67%) and summer (33%) from lactating Holstein cows in three commercial herds were included in this study. Animals were presynchronized with two injections of prostaglandin $F_2\alpha$ (PGF) given 14 d apart (starting at 26 ± 3 DIM) followed by Ovsynch (gonadotropin releasing hormone [GnRH]-7d-PG-56 h-GnRH-16 h-timed AI) or Cosynch (GnRH-7d-PG-72 h-GnRH+timed AI) 12 d later. At the time of AI, services were randomly assigned to 1 of the 2 groups: 1) with (TRT; n = 1405) or 2) without (CON; n = 1438) the use of SP. Sterile cotton swab samples were collected from the tip of the AI gun (n = 102) immediately after AI (from TRT and CON) for bacteriology. Pregnancy diagnosis was determined by ultrasonography 40 ± 5 d after AI. Swab samples revealed that the use of SP was effective in minimizing contamination of the AI gun at the time of AI in TRT (51.9%) compared with CON cows (98%; $P < 0.05$). Overall, PAI was greater ($P = 0.01$) for cows in TRT (30.1 ± 1.7%) than in CON (25.4 ± 1.9%). Results from this study suggested that the use of SP reduced contamination of the AI gun at the time of AI and improved PAI in lactating dairy cows.

Keywords: dairy cattle fertility, AI, sheath protectors

Introduction

Reproductive efficiency is a major factor for profitability in dairy herds. Artificial insemination is the most common practice used to breed dairy cows in the United States, accounting for approximately 78% of all services.[1] Accurate animal identification, semen handling, hygiene of the AI procedure, and site of semen deposition are paramount to achieve acceptable reproductive outcomes over time.[2,3] Inseminators should review the AI procedure on a regular basis (e.g., monthly) as well as their reproductive performance over time. Although the AI procedure (i.e., hygiene and site of semen deposition) is often overlooked, an appropriate and clean AI technique is recommended to optimize reproductive outcomes in dairy cows.

Protective AI cover sheaths, a rigid polyvinyl chloride tube of 30 cm in length x 0.7 cm in diameter, were developed to prevent vaginal contamination of the AI gun at the time of AI with the aim of improving reproductive outcomes.[4,5] The presence of manure around the perineum (i.e., vulva) is common at the time of AI in lactating dairy cows. The contact of the tip of the AI gun with manure present on the vulvar skin must be avoided to reduce the likelihood of introducing external contaminants (e.g., *E. coli*) into the uterine lumen at the time of AI. The effectiveness of using SP (on top of the regular AI sheaths) at the time of AI on PAI was evaluated in three commercial dairy herds. The objectives of this study were to: 1) evaluate the effectiveness of using disposable protective SP to minimize vaginal contamination of the AI gun at the time of AI, and 2) assess PAI in lactating dairy cows inseminated with or without the use of disposable protective SP.

Materials and methods

Lactating cows (primiparous = 1158 and multiparous = 1062) housed in free-stall barns from three commercial dairy farms were used in this study. All cows were presynchronized with two injections of PGF given 14 days apart (starting at 26 ± 3 days postpartum) followed by Ovsynch (OV; GnRH-7 d-PGF2α-56 h-GnRH-16 h-timed-AI[TAI]) 12 days later. Cows presenting signs of standing heat any time during the protocol received AI, whereas the remaining animals were subjected to TAI 16 hours after second OV GnRH. At the time of AI, 2843 services from lactating dairy cows were randomly assigned to 1 of the 2 groups; with (TRT, n = 1405) or without (CON, n = 1438) the use of SP. In the TRT group, the AI gun protected with a SP was introduced into the vagina; once in the cranial portion of the vagina adjacent to the cervical os, the SP was pulled back and only the AI gun was manipulated through the cervix into the uterine body for semen deposition. In the CON group, cows received AI without the use of SP. Furthermore, sterile cotton swab samples were taken from the tip of the AI gun (n = 102) after AI from both the treatment and control groups for bacterial culture. Pregnancy diagnosis was determined by ultrasonography 39 ± 3 days after AI. Data analyses were performed using GLIMMIX (PAI) and FREQ (culture) procedures of SAS.[6]

Results and discussion

Cultured swab samples revealed that the use of SP was effective in minimizing contamination of the AI gun (positive bacterial growth; TRT = 51.9% vs. CON = 98.2%; p= 0.05). The most common bacteria isolated (49%) at the time of AI was *E. coli*.[3] Regarding the bacterial density growth, the majority (67%) of the samples from the TRT group (AI with the use of SP) had light or sparse bacterial growth compared with the CON group (AI without SP); in which the majority of the samples showed heavy colony growth (71%).[3] Although the potential detrimental effects of introduced bacteria into the bovine uterus at the time of AI has not yet been reported, the bacteria may colonize the uterine lining and trigger an inflammatory response as shown in mares (postbreeding subclinical endometritis).[7,8] At the time of AI, not only semen but also bacteria and debris can be introduced into the uterine lumen and lead to chronic inflammation and decreased fertility in mares.[8] Under normal uterine conditions this physiological immune reaction, described as postmating inflammatory response, is cleared within 48 hours.[7,9,10] In lactating dairy cows, the proportion of polymorphonuclear neutrophils (>15%) immediately before and four hours post-AI were associated with poor reproductive performance.[11,12] Previous studies (using the same SP as this study) reported no improvement in conception to first services in dairy cattle.[4,5] These studies were conducted more than 20 years ago, using several AI technicians (and weekly randomization of SP), and only non-return rates to first service were evaluated. The effectiveness of SP (assigned to every other cow) at the time of AI in one commercial dairy herd using one AI technician and the same reproductive management was investigated.[3] Lactating dairy cows that were AI with the use of SP had greater proportion of PAI compared to cows AI without the use of SP.[3] Furthermore, in this field study the proportion of cows pregnant (all services; summer and spring from three dairy herds) was greater for cows in TRT (30.1 ± 1.7%) compared with CON group (25.4 ± 1.9%). According to these findings, lactating dairy cows may benefit from the use of SP at the time of AI by reducing the potential introduction of external contaminants such as *E. coli* (e.g., from perineum or vaginal origin) into the uterine lumen at the time of AI. In conclusion, these results suggest that the use of SP at the time of AI reduced contamination of the AI gun and improved PAI in lactating dairy cows. Performing a clean AI technique through the use of SP may be an effective strategy to improve reproductive outcomes in dairy cattle. Cleanliness of the whole AI procedure must become a top priority for professional AI technicians and on-farm breeders to achieve consistent reproductive results over time.

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Concepts and considerations for successful reproductive management and monitoring

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Abstract

Dairy owners have traditionally served many different roles on dairies, but with increasing size and complexity of the operations, there is a greater demand placed on their administrative abilities. To be successful, owner managers must take a stronger role as chief executive officer and establish the strategy and vision for an operation, create an appropriate work culture, hire and train workers, and decide how to best secure and allocate capital. Key components of a successful dairy operation are a strong training system so that employees know not only what is expected of them, but how to succeed and a timely monitoring and evaluation system. Timed artificial insemination (TAI) has been one of the most beneficial tools introduced in recent years for improving reproductive performance. There are a variety of TAI options for herds but each is designed to synchronize ovulation sufficiently to allow for acceptable conception risks while improving breeding efficiency. Herds that have experienced success with TAI have been those with excellent management and an eye for detail. Careful selection of the appropriate protocol, followed by consistent implementation and routine monitoring of both the processes and the ultimate outcomes are all key components for success. When performance is not as expected, excellent quality records that are properly evaluated can provide clues as to the potential problem areas.

Keywords: Timed AI, monitoring, reproductive management, records

Introduction

As the U.S. dairy industry continues to consolidate into fewer, but larger farms, the demands for effective management and leadership skills have increased. Owners have historically run their dairies via a hands-on approach, providing much of the labor necessary; but with increases in herd size and efforts to maximize the return on their milking parlor by milking around the clock, owners have had to make the transition to more of a role as administrator and supervisor. With increased herd size has also come a more specialized labor force, often with very defined roles of highly technical tasks. With this expansion, an increasingly detailed ability to capture and evaluate day-to-day activities and results has developed. Consequently, the need for more effective and timely abilities to monitor, manage, and provide feedback has dramatically increased.

As milk production per cow has increased, there have been some challenges with achieving good reproductive performance. Physiologically, there are significant differences between Holstein dairy cows producing large volumes of milk and those producing below average volumes and these differences include a reduction in the duration of estrus, decreased estrus detection efficiency, potential declines in conception risk (CR, defined as the percentage of matings that result in a diagnosed pregnancy [i.e., the number of new pregnancies diagnosed divided by the total number of cows inseminated]) as a consequence of prolonged luteinizing hormone stimulation and delayed ovulation, as well as declines in CR due to inaccuracies around estrus detection.[1,2] However, well managed herds have been able to continue to get cows pregnant in a very timely manner by reducing the impact of metabolic and uterine health challenges and by placing more emphasis on a well-structured reproductive program.

In an effort to improve reproductive performance, a number of new tools and technologies have been developed and research is constantly on-going. The use of TAI protocols has mitigated much of the impact of high milk production and feed intake on the ability to detect estrus and inseminate cows in a timely manner. Technology in the form of activity monitoring systems and radio frequency identification (RFID) can help to reduce the risk of human error and improve compliance within the breeding management program when implemented and used appropriately. In addition, there has been a shift away from placing genetic selection pressure predominantly on milk production to increased selective pressure for other important traits including reproduction. Each of these changes places an additional burden on management; dairy managers must hone their economic decision making skills to select the correct

technology to match their herd's abilities and goals; they must work to provide adequate employee training and supervision; and they must devise proper monitoring approaches to assess the potential profitability of these tools and the goodness-of-fit for these tools within their dairy.

No therapeutic intervention or new technology can compensate for poor management, a lack of appropriate training, or for inaccurate record keeping. It is vital for today's modern dairy to employ high quality management and employees and to ensure they are well trained and efficient in all aspects of production. Timely and accurate records will allow for identification of areas of excellence and improvement, thus increasing the efficiency and economic viability of the enterprise.

Evolution of timed AI protocols

For many herds, the use of TAI has reduced or eliminated the need for estrus detection when implemented with an appropriate voluntary waiting period and good animal health monitoring practices and has provided economically positive results for most herds, despite some of the management challenges. Initial implementation of a TAI program may result in a number of difficulties including labor and facility management (can the workers safely and adequately restrain the correct cows without creating undue stress in the cows?), injection compliance (did the correct cows receive the correct injection on the correct day?), and accurate record keeping (was the correct information entered into the records system accurately and timely?).

In the authors' opinions, the widespread adoption and implementation of TAI has been a large contributor towards the improvement in reproductive performance that appears to have occurred in the past few years. During the late 1990's, two large data sets, one from California containing 80 herds and 100,000 cows and one from the upper Midwest containing over 2,200 herds and 250,000 cows, were assembled.[3] In these reports, the average pregnancy rate (PR), defined as the percentage of eligible cows that became pregnant within a given time frame (usually 21-d), was 14-16%. During the last ten years, herd numbers have decreased, herd size has increased, adoption of TAI and other reproductive technologies has increased, and the general consensus is that dairy cattle reproductive performance has improved. Now, our expectations are that herds should have PR of at least 18-20% with many Holstein herds achieving high levels of milk production while maintaining annual PR of 24-26%.

Each of the TAI approaches (see Figure 1) has its own advantages and disadvantages including differences in efficiency at recruiting cows into ovulation cohorts and the number and type of injections required and utilization of TAI may allow more efficient use of labor while also providing improved reproductive efficiency. The choice of whether to use a TAI program and of which program to use should be determined by the management's assessment of labor, facility, and cow health constraints as well as the current level of reproductive performance. Each of the successful TAI protocols is based upon some variation or derivative of Ovsynch, as shown in Figure 1.[4] Conception risk for Ovsynch typically ranges from the upper 20's to mid-40's, depending upon the cyclicity status of cows at the start of the program, the presence or absence of some presynchronization strategy, the timing of the interval between the prostaglandin $F_2\alpha$ (PGF) injection and the second gonadotropin releasing hormone (GnRH) injection, and whether the Ovsynch is for first service or as part of a re-synchronization approach.[5-9]

Many herds utilize once-daily estrus detection during the morning hours via the use of visual assessment of tail chalk status and will inseminate at this time. One frustration regarding Ovsynch for some dairies is the requirement for either an afternoon injection or an afternoon insemination, depending on the program version used. Administering the GnRH in the afternoon at 56-60 hrs after PGF shifts the insemination back to the morning period and slightly improves CR as compared to the morning injection of the second GnRH at 48 hrs, followed by an afternoon or evening insemination in 12-16 hrs.[10] In order to eliminate some of the compliance issues associated with Ovsynch, Cosynch-72 was developed (Figure 1) with each of the steps performed during the morning breeding management period, facilitating greater potential compliance. However, the anticipated CR for Cosynch-72 is expected to be lower than that for Ovsynch.[6]

Another variation to the traditional Ovsynch program has been the insertion of a progesterone-releasing intravaginal device (CIDR®, Pfizer Animal Health, New York, NY) into the protocol starting at

d-0 (Figure 1). Cows that begin the Ovsynch protocol without a corpus luteum present, whether due to being in an anovulatory condition or due to starting the protocol in metestrus or proestrus, have a reduced expected CR. However, the addition of the CIDR, a vaginal drug insert containing 1.38 g of progesterone, has been shown to improve the CR in these animals by 5-10%.[11]

Ovulatory response to the first injection of GnRH of Ovsynch is a very critical determinant for successful synchronization of ovulation in dairy cows and as a consequence, various presynchronization options have been developed. A presynchronization protocol utilizing two injections of PGF given 14 days apart and 10-14 days prior to starting Ovsynch (Presynch-Ovsynch, as shown in Figure 1) has proven very successful for many herds.[12] In addition to improving the consistency of the ovulatory response to the first injection of GnRH of Ovsynch in cycling cows, other benefits include an improvement in uterine health and the ability to "cherry pick" cows and breed them via estrus detection following PGF administration.[13] With a Presynch-Ovsynch program, cows may be inseminated at the detected estrus or be started into an Ovsynch protocol 10-14 days after the second PGF injection, but optimal results appear to result from the use of an 11-d interval.[8,12,14,15]

Alternative presynchronization strategies that utilize PGF and GnRH are Double-Ovsynch and G6G (Figure 1). Protocols using GnRH such as these will increase the ovulatory response to the first GnRH injection of Ovsynch similarly to presynchronization with PGF, but have the added benefit of potentially improving CR in cows that begin TAI as anovulatory.[16,17] The downside risks of these GnRH-based presynchronization protocols are the decreased ability to enhance uterine health and the additional complexity of either additional injections or of the administration of injections on inconsistent days of the week.

Herds with high reproductive efficiency continue their efforts beyond first service and may also utilize program such as Ovsynch, Co-Synch-72, and CIDR synch for re-synchronization efforts. Resynch is the term used to describe the use of Ovsynch to re-synchronize ovulation in cows following an insemination and the optimal time to initiate Resynch is at d26 or d33 following the previous insemination.[18] This program can be utilized in any AI herd regardless of the approach used for pregnancy determination since cows may be enrolled into a Resynch program at the time of pregnancy evaluation or 7d prior to scheduled pregnancy determination. If enrolled 7d prior to pregnancy determination, all cows receive the GnRH, but only those determined to be non-pregnant a week later receive the PGF and complete the protocol. This re-synchronization strategy allows for a more rapid re-insemination of non-pregnant cows that are not observed in estrus.

Hallmarks of successful management

Effective managers have a variety of ways of making good things happen through people. The specific style may vary by individual, but in general, there are a few important characteristics of successful dairy management.[19] First, successful dairy management should be based on biologically and economically sound principles that will take advantage of proven strategies. Few people today question the wisdom of feeding a total mixed ration, using AI sires, or adopting safe and effective preventive health approaches such as vaccines, but good managers understand how to select the correct mixer wagon for their operation and when to replace it; they understand the principle of genetic improvement through careful selection of a portfolio of sires; and they understand that while they may not grasp all of the principles of immunology, their herd veterinarian can help them design and implement a strategic vaccination program to help manage risk.

Second, good dairy management focuses on promoting responsive interpersonal relationships, strong people skills, and provides adequate training to enable employees to correctly and efficiently complete their job. Well managed dairies help people grow and develop their skills through appropriate and timely education and on-the-job training. For example, if a dairy herd wants to improve their management of lameness, one approach is to identify a candidate for hire who has the appropriate training or to send an employee to a hoof trimming training school in order for that individual to develop the appropriate skills. In this case, the manager has invested time and resources into improving the skills of an employee that

will likely lead to improved herd performance. Subsequently, management should determine the impact of this training program and provide appropriate and timely feedback on how the employee is performing.

Third, successful dairy management will adopt and implement new protocols or procedures which have a high probability of success and avoid unnecessary complexity. A great example of this concept is the adoption of TAI. Moving a herd that has never used TAI, nor had to find cows and give scheduled injections, from an estrus detection-based breeding approach to a very complicated TAI protocol such as Double Ovsynch with scheduled activities on four different days of the week is very risky due to the complexity of the protocol and the critical importance of complete compliance. While the TAI program has the ability to consolidate labor into discrete chunks of time, it still carries significant risk and other options such as a Presynch-Ovsynch program which still allows for the utilization of estrus detection after the second PGF injection may be easier and less risky for many herds.

Dairymen routinely make decisions in a risky and often, uncertain environment. Astute management will carefully evaluate new opportunities for investment or implementation in their operation and adopt new technology, but only after careful consideration of the risks versus rewards. Correct and profitable decisions are made when a dairyman chooses to use a certain product or technology and it delivers a profitable response; or when he decides not to use a product or technology because in actuality, it would fail to deliver a profitable response, at least in his operation. Conversely, an incorrect economic decision is made (type I error) if the producer chooses to use a product or invest in a technology which does not deliver the expected profitable response. If he chooses not to use the product or technology when in reality it would have made him money, he has incurred lost opportunity cost by failing to use it and thus, made a type II error.

Effective and successful managers work to reduce variation within the system in order to more clearly assess performance trends; when changes are made via the addition of a new product, technology, or implementation of some protocol, good managers determine up front how it will be monitored and evaluated. For example, with the adoption of a TAI protocol, a new breeding code should be created to differentiate these inseminations from those based on estrus detection. Herd management software programs such as DairyComp 305® (Valley Ag Software, Tulare, CA) allow for multiple breeding and technician codes so changes in reproductive management and performance can be easily measured and evaluated. Of course, timely input of data is critical to monitor performance and high quality operations will have one or more people dedicated to the entry of farm data in a timely manner.

Finally, successful dairy management requires good personnel management and "people" skills. The ability to effectively coach employees and communicate with them and is often the major difference between a top dairy and an average one. Employees need timely feedback regarding their performance and effective managers carefully critique their workers in an impartial and confidential way. Timely performance evaluations, reflecting both the positive and the negative, are critical for helping employees grow in their confidence and ability, and performed properly, will help them become more important parts of the team. Equally critical is the importance of verifying that the apparent results or outcomes attributed to an employee are truly valid, accurate, and the consequence of that individual's work.

Changing approaches to monitoring and management of reproduction

In order to gauge the progress of a dairy, proper and timely recording and evaluation of key data are essential. Dairy reproductive monitoring involves the regular observation and recording of activities, events, and outcomes that occur for the purposes of observing and evaluating the degree of change, intended or unintended, positive or negative, within a herd. It should include a systematic approach to data collection, evaluation, and provision of feedback about the changes detected. Routine and systematic monitoring should allow for the recognition of "normal" performance; should aid the herd manager in evaluating the impact of intentional change in management or performance; should facilitate the discovery of unintended drifts or declines in procedures or performance over time; and it should help determine the potential causes or sources of abnormal performance.

Goals are target levels of performance toward which producers are trying to achieve and every operation should have simple, specific, measurable, and timely goals. However, it is rarely a good idea to

use the goal metric itself as a monitor of performance since it usually represents the end result of many different processes. For example, consider a herd that has an average age-at-calving for replacement heifers of 27 months. Most herds have a goal for age-at-calving of 24 months or less. Using the herd's current age-at-calving to establish a reasonable goal is appropriate, but relying on it as a monitor of changes in performance is very problematic since it is the cumulative result of many processes such as appropriate feeding, housing, vaccination, breeding, culling, etc. Changes in any of these areas today would not result in a measurable change in age-at-calving for months. A manager could cull out the oldest, poorly growing heifers at some point prior to calving by selling them to his neighbor, thus reducing his average age-at-calving, but he really hasn't changed the true performance of his replacements. Conversely, he could initiate dramatic changes to the feeding and management of his youngest calves, but the impact of this change will not be measured using this metric until approximately two years into the future.

Evaluating a system such as heifer reproductive management by examining the age-at-first calving utilizes an animal-based outcome; but the results are a consequence of animal performance (response to the housing and nutrition program, genetic potential for growth and fertility, breed, etc), management's efforts (level of metabolizable protein and energy provided at various stages of growth, promptness of movement of animals into an AI pen, training or hiring of breeding technicians, sire selection, etc), and worker competence and adherence to the management plan (delivery of the proper feed to the correct pen, correct and timely identification of animals in estrus, semen handling, adherence to TAI protocols, proper identification and recording of procedures, etc). Whenever possible, the focus for performance analysis should be on monitoring the key steps of the process versus simply monitoring outcomes. While outcome monitoring is important and has its place, monitoring key processes and labor's contribution to these processes gives an earlier indication of problems or improvements, thus allowing more timely correction or celebration.

A critical yet often overlooked issue surrounding TAI, and other reproductive management technologies such as activity monitoring, is the importance of training and compliance. Dairy owners/managers must take a key lead role in order to optimize returns and reduce the risk of failure when adopting one of these technologies. Focus should be directed at training farm personnel as to *who* needs to do *what*, *when* do they need to do it, and *how* they should proceed to get it done. In an attempt to monitor employees, managers often try to devise clever ways to catch employees doing something wrong instead of working to help enable them to do things correctly. A great example is the practice of giving an employee a list of cows due to receive an injection and including some "dummy" cows that do not exist just to see if the employee will check these cows off the list along with the cows that actually received the injection. While this approach will serve to catch the employee that is more interested in the *appearance* of successfully completing a job, a completed checklist is no indication that he gave the correct injection to any cows; only that he falsely reported giving injections to cows that did not exist. Unfortunately and far too often, the only interaction an employee has with an owner/manager is when he is scolded for failing to do a task correctly.

Instead of merely devising ways to catch employees failing, management should focus on an active and ongoing training process that includes the following five points: Explain, Show, Practice, Observe, and Praise.[20] Managers should explain to employees the TAI protocol and why each individual step is important. Care should be taken not to overload them with a complicated lesson in physiology, but rather to provide a more simplistic overview of the purpose of each component of the protocol. They should demonstrate each step in the process and how to best complete each task and then allow the employee the chance to practice each step. In the case of teaching TAI, managers should have an employee demonstrate how they will draw up the required amount of GnRH and then to administer it to a cow; observe how the employee performed the task; and repeat the previous steps as needed to ensure that the correct product is administered accurately. Was a 1.5 inch 18 ga needle that was attached to an appropriately sized syringe used to administer the GnRH in an appropriate muscle plane? If so, the manager should praise him for a job well done. If not, he should praise him for the steps performed correctly and then help the individual to modify the incorrect ones.

A variety of approaches and details on how to monitor and evaluate reproductive records have been described elsewhere.[21-24] Today's computer records programs have the ability to create many different graphs and figures. Unfortunately, many people settle for the pre-formed graphs or canned reports and try to determine what these reports are telling them. However, the population at risk is often not defined, the time at risk is not specified, or the metric reported does not necessarily reveal the true condition of the system. A better approach is to use a few general reports or graphs that describe a few important outcomes of interest as screening tools. Then, ask more specific, appropriate questions, preferably related to the processes rather than simply the outcome, and find the data that answers the questions posed. With this approach, the operator must first think of the question (i.e., the problem) and look to find answers using specific ranges in time.

Selecting the appropriate items to record for monitoring is important; do not ask employees to collect mountains of data simply for the sake of collecting data unless there is a plan to use it. Once it is collected, use the information to provide feedback, both positive and negative, to employees. Otherwise, with a lack of feedback, data collection deteriorates, the quality of both the records and the work declines, and the data become useless for monitoring.

When evaluating reproduction, there are a few key questions that should serve as the basis for investigation, with additional questions developed as the investigation continues, depending on the initial outcomes.[24] It is beyond the scope of this paper to completely describe an approach to investigating reproductive performance through computerized records review, including the important component of validating the accuracy and completeness of the data. Instead, one potential approach towards investigating potential issues as they relate to compliance and reproductive management of TAI protocols will be detailed. For the purposes of this paper, the DairyComp 305 records from several large dairy herds milking between 1500 and 3000 cows each will be utilized to illustrate specific examples. Each of these herds utilizes TAI to varying degrees and is located within the U.S.

When investigating reproductive performance, one of the first areas to examine is the days-to-first-insemination graph to assess the voluntary waiting period, to try and understand their approach to first service, and to assess the consistency with which cows have been presented for first service. In Figure 2, the first insemination history for the past year for Herd A, a 1900-cow Holstein dairy is shown. Each colored square represents a cow and the y-axis is the DIM at first insemination and the X-axis reflects the current DIM for cows in the herd. The colored squares at lower left are cows that have not yet been inseminated. Based upon this figure, Herd A is utilizing some form of TAI (Double-Ovsynch) with a very high level of compliance since very few cows are inseminated outside of the 1-week range starting at the voluntary waiting period of 70 DIM for first service. Table 1 is the historical reproductive performance for this herd for the first five 21d cycles following the voluntary waiting period for the past year and it shows the net impact of this high level of compliance and a high first service CR. This herd is a Southeastern dairy that has adopted the use of RFID and takes great advantage of this tool with an annual PR of 26%. Radio frequency identification is a system that wirelessly transmits information from a transponder (ear tag) to a hand-held reader using radio waves. The advantage of this system is that there are no mis-read numbers, no missing cows (assuming all tagged animals are scanned), and a faster ability to locate animals for injections, vaccinations, examination, etc. Based upon the computerized records, 97% of all cows on Herd A are inseminated for the first time between 70 and 76 DIM with 99% serviced between 70 and 90 DIM, thanks in large part to the excellent compliance afforded by the RFID system.

For comparison, consider Herd B, a Southwestern dairy that utilizes a commercial reproductive management team that relies heavily on ultrasound to make individual cow-based breeding decisions. The herd is set up on a Presynch-Ovsynch program, but individual decisions are made for each cow based upon findings at each weekly ultrasound. Instead of achieving 95% insemination efficiency or more within the first week of the voluntary waiting period as is expected with a weekly TAI program, this herd breeds 43% within the first week. This program was implemented in an attempt to maximize CR but suffers from compliance challenges and delays in actually delivering inseminations. Figure 3 shows the first insemination history for Herd B. This herd has ten different recorded breeding codes for first service, and despite all of the weekly ultrasound examinations and individualized decisions for guiding

the inseminations, the first service CR is only 32%. Table 2 reports the annual PR results for the first few 21d cycles of breeding in a similar layout as Table 1. Based upon the annual PR of 13% and the poor overall results within the first few cycles, it is quite clear that Herd B struggles to get cows inseminated in a timely and efficient manner. By delaying some inseminations, only 82% of cows are inseminated in the first 21d cycle. This herd's reproductive performance suffers from its very complex breeding system and might benefit from simply placing cows onto a more regulated Presynch-Ovsynch or Double-Ovsynch program for first service instead of delaying completion of the TAI program based on ultrasound findings of the ovaries.

Another key figure that may be useful in terms of assessing overall herd compliance issues is consistency with which the herd's management team presents cows for pregnancy evaluation. In DairyComp 305, this graph is created using the following command: "GRAPH PREG BY DSLH\W1". Figure 4 shows the days since last insemination for the first pregnancy determination (35 to 41 d since last insemination) and for the verify exam (71 to 77 d since last insemination) for Herd A. The Y-axis is a count of events and the X-axis is the days between insemination and pregnancy determination for either the first pregnancy determination or the pregnancy verification (second pregnancy examination for pregnant cows). Not pictured here is the final pregnancy examination that occurs between 211 and 217 days after conception. Notice the scarcity of data points after each set of pregnancy examinations. This herd conducts pregnancy evaluations once a week and the few points that appear out of the schedule are most likely cows that were flagged for re-checks due to issues detected by palpation per rectum. The highest columns correspond to cows inseminated via TAI.

For comparison, consider Herd C's graph shown in Figure 5 using a slightly different command string. Again, the X-axis represents the number of days since insemination for the pregnancy examination and the Y-axis is a count of cows. Herds A and C both utilize only AI for first service and both herds utilize a veterinarian for pregnancy determination on a weekly schedule. The major difference between the two herds is that Herd A uses RFID to find all cows on the list and will seek out cows that are missing while Herd C uses a paper list and has the philosophy of "well, we'll get her next time" if she is not found during the herd check. To better compare the two herds, consider Table 3, which contains a breakdown of the days-since-conception at pregnancy evaluation for each herd. Herd C has approximately ten times greater odds of having cows experience a delayed pregnancy evaluation as compared to Herd A. If all cows were pregnant at examination, there would be no issue; but when cows are skipped and later found to be non-pregnant, the herd has experienced an economic loss via a lost opportunity to intervene with a strategy to deliver the next insemination. Also, an attitude such as the one shown by Herd C is likely to carry over into the administration of injections for TAI protocols, delivery of vaccinations, movement of cows, etc. Depending on where the cow is in her injection schedule, a single missed injection could be the difference between a pregnancy and another 40 or more days open, as well as the wasted injections.

Another area that should be examined when investigating reproductive performance, and TAI specifically, are the results of the inseminations, including the CR by breeding code and whether or not the results match the intent of the reproductive program. Table 4 shows the average CR for the past year for three herds, as well as the service-specific CR by breeding code for the first and second services. Herd A's goal is to breed all cows for the first time using the Double-Ovsynch protocol. After first service, cows are examined daily for estrus and bred accordingly. If not observed in estrus within 28-34 d, cows receive a GnRH injection as part of the re-synchronization protocol. Pregnancy examination occurs at 35-41 d after previous service and non-pregnant cows then receive a PGF injection and complete the Ovsynch protocol with the next service occurring at 45-51 d since the previous service. In this herd, 100% of the first inseminations are via Double-Ovsynch and 55% of the second services are via estrus detection, with the remainder occurring via Ovsynch. Based upon the authors' clinical experiences, the CR for each of these services is above average for herds in the Southeast. One concern that some may express is that only 55% of the second services were performed via estrus detection. However, considering the re-synchronization approach used in this herd, cows have only one opportunity to recycle with a normal return to estrus prior to starting the Ovsynch program for the second service. In addition,

there is a conscious decision to not inseminate cows that display marginal signs of estrus and instead, allow them to pass through to the Ovsynch program. As a result, both of the second service approaches yield very acceptable levels of CR and yet, are performed in a timely manner.

In comparison, consider the CR results for Herds F and C that are shown in Table 4. These two herds are Western herds that utilize a Presynch-Ovsynch program with TAI occurring by 80 DIM, but inseminators are strongly encouraged to breed cows following the PGF injection prior to starting Ovsynch. However, the results are quite different between these two herds. Herd F does a very good job aggressively identifying cows in estrus and does not appear to experience significant issues with anovular cows based upon the very acceptable first service CR with both breeding approaches. Herd C, however, suffers from cyclicity challenges, uterine health issues, estrus detection accuracy problems, and an inseminator that feels threatened by the TAI protocol. This inseminator begins breeding as early as 40 DIM and manages to inseminate 94% of the cows via estrus detection for both the first and second service opportunities. A word of caution is warranted here; the estrus detection efficiency is only about 60% for each cycle, but since there are almost two full cycles at risk for estrus detection prior to the TAI, a high proportion of cows never make it to the TAI. Normally, this would be a good thing, but in this case it is not. In Herd C, the inseminator breeds a lot of cows showing marginal signs of estrus. If the breeder applied a little more scrutiny to each cow and inseminated fewer animals via estrus detection, chances are good that the CR to both estrus detection and TAI would improve and the overall PR would increase.

Space does not allow the full explanation of how to completely evaluate CR, but in addition to examining it by service number, breeding code, and interval length since previous service, CR should be evaluated by parity, technician, day-of-the-week, month, and by combinations of these factors. However, with each additional strata, the sample size gets smaller and our ability to accurately interpret binomial outcomes such as CR decreases. In Herd F that was mentioned in Table 4, the reason that the confidence intervals around the CRs were so small was because there were approximately 50,000 total inseminations in this individual herd in one calendar year. Most herds do not have enough cows to achieve this much statistical power and extreme caution should be practiced when evaluating reproductive results.

Another potentially helpful tool within the computerized records for evaluating breeding management, compliance, and cyclicity status is to examine the interval between first and second inseminations while controlling for the first service breeding code. The normal expected interestrus interval for dairy cattle is 18 to 24 d. However, many factors can impact the actual interval to next service including accuracy of estrus detection, technique used for first service (TAI or estrus detection), cyclicity status immediately prior to first service, compliance to TAI protocols, stage of the cycle when starting TAI, and pregnancy wastage (early or late embryonic death or abortion). If cows are incorrectly identified as in estrus and inseminated, the return to next service should be less than or equal to 18 to 24 d, depending upon when in her cycle she was first inseminated. If early embryonic loss occurs (a loss of a pregnancy prior to approximately d17 of the cycle), a normal return is expected. However, if late embryonic death occurs (loss of a pregnancy after d17 but before d42), the return to estrus is expected to be lengthened and the actual return depends on when the loss occurs and the status of follicular development at the time of the loss.

When TAI is involved in the first service, a variety of unusual intervals are possible, depending upon whether or not a presynchronization series was used, whether all injections were given as required, and whether the cow was cycling or not prior to starting TAI. For example, consider Table 5, a breakdown of the interestrus intervals between first and second inseminations for three herds utilizing different approaches to delivering first insemination. At the top of the table are some commonly used goals for herds relying on estrus detection, but these goals are not applicable to herds relying heavily on TAI. Herd A utilizes a Double-Ovsynch program for all first services; Herd D uses estrus detection and prides itself on very high estrus detection efficiency; Herd E uses the Presynch-Ovsynch protocol to synchronize cows for first service, but inseminates any cow observed in estrus following the second injection of PGF within the presynchronization series and then delivers a TAI insemination to those cows not previously inseminated.

Herds utilizing TAI have a different set of expectations for the intervals from first to second service as compared to herds relying on estrus detection. For TAI herds utilizing some form of presynchronization prior to Ovsynch and with a low prevalence of anovulatory animals at the start, there should be fewer than 1% of cows in the d1 to d3 category, since any cow that would express estrus within three days of a TAI should have had a follicle capable of responding to the final GnRH if the injection was properly given. The only cows that are expected to actually re-cycle within this time period would be cows started on Ovsynch on d3 or 4 after ovulation (Paul Fricke, personal communication). However, in herds that do not presynchronize prior to TAI, assuming that cows are randomly distributed throughout a typical cycle, about 9% of the cows would start Ovsynch on d3 or 4 after estrus and therefore, on average about 9% would be expected to have first to second service intervals of 1-3 d. These cows fail to respond to the first GnRH injection, but do respond to the PGF injection seven days later; these animals are likely to have a new follicular wave starting that is not yet capable of responding to the final GnRH injection. Consequently, the cow expresses estrus two to three days after the TAI.

Several factors related to TAI will alter the proportion of cows appearing to have first-to-second service intervals of 4 to 17 d. First, when cows are still anovulatory at the start of the Ovsynch protocol, some will ovulate for the first time in response to the final GnRH injection. Since this is the first time that her body has experienced a rise in progesterone since parturition, she is likely to experience a shortened luteal phase followed by spontaneous luteolysis and may express estrus anytime from d8 to d12 following the first TAI. Careful evaluation of the first-to-second service interval can thus be used to suggest that cyclicity issues may be a problem in herds relying heavily on TAI for first service. Second, cows that start Ovsynch in the second half of the estrous cycle that fail to ovulate to the first GnRH will come into estrus during the protocol, usually around the time of the prostaglandin injection. If these cows are not serviced at this time based on the expression of estrus, they will appear to have a shortened interestrus interval of 14 to 17 d, but in fact, have actually had a normal cycle.[25] If cyclicity and presynchronization are not problematic, there should be less than 5% of cycles falling in the d4 to d17 first-to-second service interval. If this number rises, more scrutiny into the actual distribution within this category is required to determine the potential cause.

Aside from using secondary indicators of cyclicity and compliance such as the herd breeding records, perhaps a better, albeit more expensive, approach would be to sample a number of cows twice to measure progesterone levels via serial sampling approaches or to perform an ultrasound examination.[5,11,12,26] With ultrasound, if cows have been presynchronized, examinations can be performed at the time of the first GnRH injection since cycling, presynchronized cows should have a functional, measurable corpus luteum at this time. Using progesterone testing, samples can be taken at the time of the final PGF injection of presynchronization and again 10 to 14 d later when GnRH is first administered. If blood progesterone levels at one or both of the sampling times is $\geq$1.0 ng/ml, the cow is classified as cycling.

If the objective is to measure TAI synchronization efficiency, a slightly different approach is taken. For this purpose, a blood sample is taken from 20-25 cows on the day of TAI and again from the same cows 7-10 d later. If the cow was cycling and the PGF injection was given appropriately, the progesterone level at the time of TAI should be less than 1.0 ng/ml. In 7-10 d, progesterone should be elevated above 1.0 ng/ml. If both samples have progesterone levels below 1 ng/ml, the cow is not cycling. Notice that this approach is called synchronization efficiency and not synchronization compliance. Without multiple repeated samples from the same cow, there is no way to truly assess compliance for each injection. However, if progesterone is high at the time of TAI, cows will not become pregnant and there was obviously a failure of cow identification or failure of appropriate administration of an adequate dose of PGF. A generally cited goal for this measure of efficiency is 90-95% (Neil Michael, personal communication).

Summary

In years past, dairy owners often wore many hats: lead milker, primary feeder, maternity manager, breeding technician, hospital pen manager, and crop supervisor just to name a few. Modern dairies, however, have dramatically increased in size and complexity and most successful owners have transitioned to more of a "chief executive officer" or similar role, setting the strategy and vision for an operation, creating an appropriate work culture, hiring and firing workers, and deciding how capital will be secured and allocated. In order for dairies to achieve top performance, each specific area of the dairy–calf rearing, milking, feeding, breeding, etc–must perform well since problems in one area will sooner or later impact another area and threaten the overall economic viability of the unit.

An effective owner/manager establishes a business plan and organizes the system in a way to optimize its likelihood for success. This leader develops and trains workers that are willing and able to perform their jobs satisfactorily. A key component of this system is a strong training system so that employees know not only what is expected of them, but how to succeed. The proper tools to allow them to succeed are provided; and an appropriate system for capturing and recording data regarding herd and labor performance that will facilitate prompt and accurate monitoring of performance is developed.

One of the tools that successful managers have used to improve reproductive performance in their dairy herds has been TAI. A wide variety of protocols exists, but each one is designed to synchronize ovulation in order to afford an acceptable CR via appointment breeding. One of the clear differences between herds that have been successful with TAI and those that have failed has been the attention paid to compliance to the protocol. When utilizing TAI, careful selection of the appropriate protocol, followed by consistent implementation and routine monitoring of both the processes and the ultimate outcomes are all key components for success.

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Figure 1. A schematic representation of some of the predominant TAI protocols in use on dairies around the world.

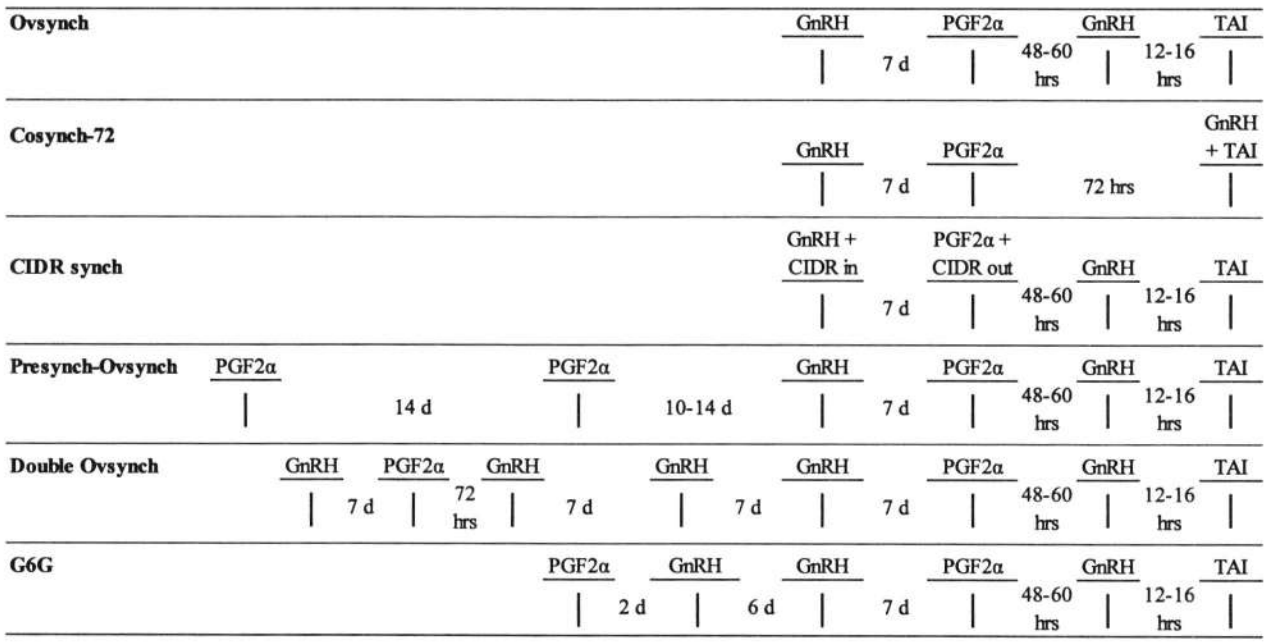

Figure 2. A scatterplot of days-to-first insemination graphed by current DIM for a 1900-cow dairy utilizing Double-Ovsynch for first service.

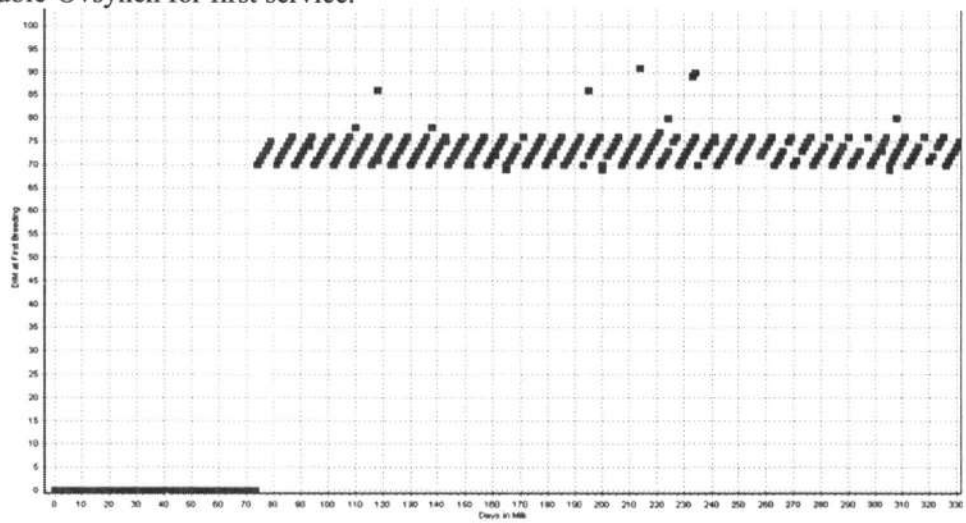

Figure 3. A scatterplot of days-to-first insemination graphed by current DIM for a large Southwestern dairy utilizing an ultrasound-based breeding management service and TAI for reproductive management.

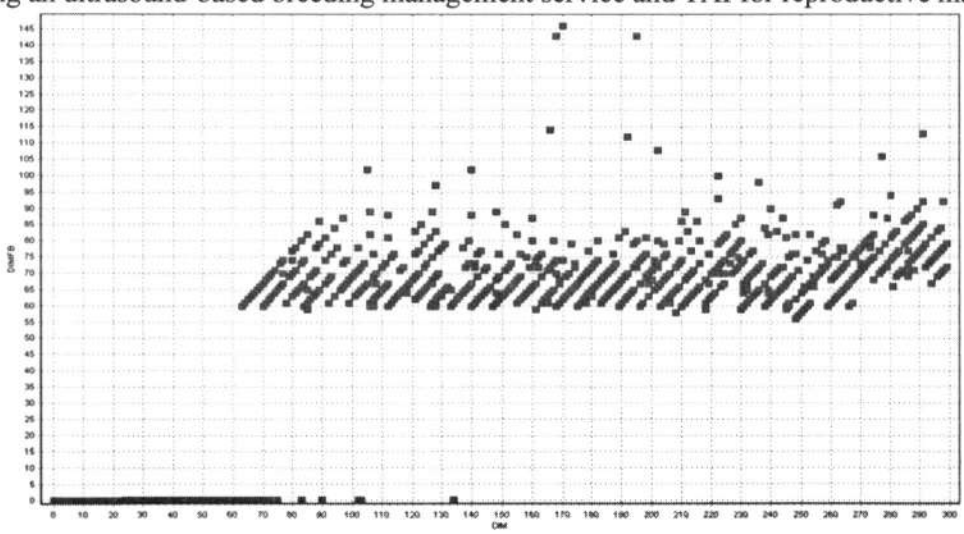

Figure 4. A frequency histogram of the days from breeding to the first and second pregnancy evaluation for cows in Herd A.

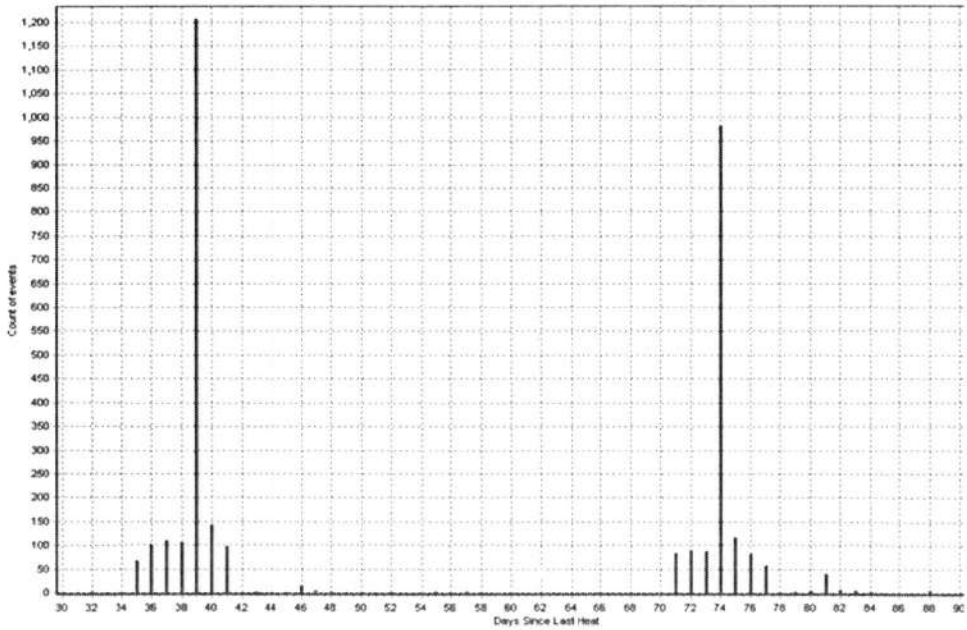

Figure 5. A frequency histogram of the days from breeding to the first pregnancy evaluation for cows in Herd C, a 3000-cow Western dairy that utilizes palpation per rectum for pregnancy evaluation and weekly herd visits.

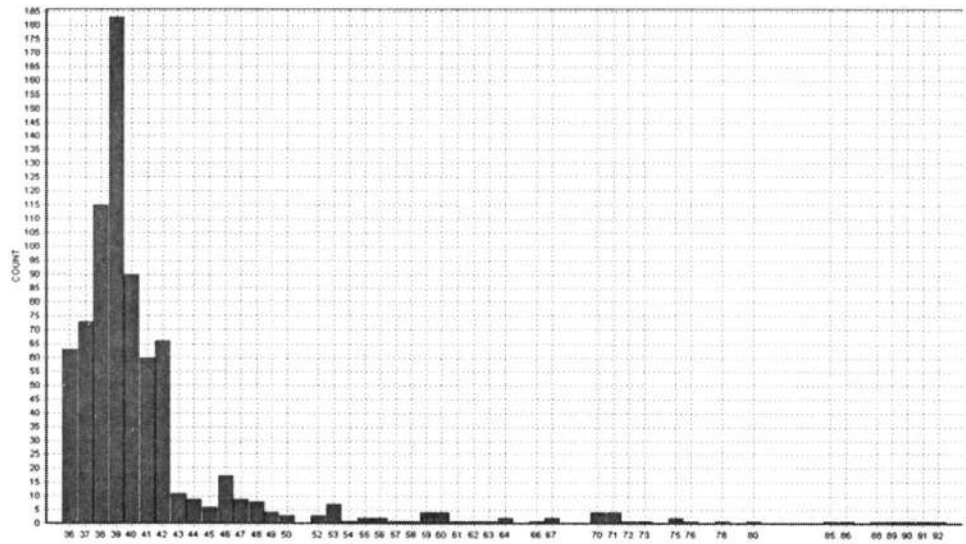

Table 1. A snapshot of the first 5 21-d cycles in the PR report, arranged by DIM, for a 1900-cow Southeastern dairy utilizing Double-Ovsynch for first service and RFID. The initial DIM at the start of each breeding cycle is shown in the left column (DIM) and the PR results within a row are calculated by dividing the number pregnant (Preg) by the number considered eligible for pregnancy (Pg Elig).

DIM	Br Elig	Bred	Pct	Pg Elig	Preg	Pct
70	1278	1265	99	1225	507	41
91	694	313	45	688	121	18
112	528	450	85	489	142	29
133	352	199	57	347	65	19
154	294	220	75	275	67	24

Table 2. A snapshot of the first 5 21-d cycles in the PR report, arranged by DIM, for a 1800-cow Southwestern dairy utilizing Ovsynch for first service.

DIM	Br Elig	Bred	Pct	Pg Elig	Preg	Pct
60	1812	1487	82	1788	424	24
81	1346	516	38	1331	135	10
102	1193	739	62	1175	152	13
123	960	471	49	941	125	13
144	826	453	55	816	99	12

Table 3. Breakdown of days-since-conception at pregnancy evaluation for two herds with different attitudes towards the importance of finding all cows on the list.

	Days at Pregnancy Evaluation		
	< 35	35 - 41	≥ 43
Herd A (percent)	0%	98%	2%
count	0	1052	19
Herd C (percent)	0%	84%	16%
count	0	650	123

Table 4. The average conception risk (and 95% CI) and service-specific conception risk stratified by breeding code for the first and second service over the past year for three herds with different approaches to reproductive management.

Herd A	All Services	1st Insemination	% of services	2nd Insemination	% of services
Estrus Detection	33% (30-37)	-	0	39% (33-45)	55%
Double-Osynch	43% (40-46)	43% (40-46)	100%	-	0%
Ovsynch	32% (29-35)	-	0	31% (26-37)	45%
TOTALS	37% (35-38)	43% (40-46)		36% (32-40)	

Herd F	All Services	1st Insemination	% of services	2nd Insemination	% of services
Estrus Detection	37% (36-38)	41% (40-42)	82%	43% (42-44)	91%
Ovsynch	31% (30-32)	34% (32-35)	18%	29% (27-31)	9%
TOTALS	36% (35-37)	40% (39-41)		42% (41-43)	

Herd C	All Services	1st Insemination	% of services	2nd Insemination	% of services
Estrus Detection	28% (27-30)	29% (27-31)	94%	30% (27-33)	94%
Ovsynch	24% (20-28)	25% (19-31)	6%	24% (15-33)	6%
TOTALS	28% (26-30)	28% (27-30)		29% (27-32)	

Table 5. A stratification of interestrus intervals between the first and second service of three dairy herds utilizing different approaches to reproductive management.

	1 - 3	4 - 17	18 - 24	25 - 35	36 - 48	> 48	
Common goals with estrus detection	< 5%	< 10%	> 40%	< 15%	< 15%	< 10%	
Herd A	1%	5%	33%	12%	48%	1%	
(Double-Ovsynch)	13	43	300	112	445	6	Total 919
Herd D	6%	10%	58%	11%	11%	3%	
(Estrus Detection)	56	93	536	105	99	28	Total 917
Herd E	1%	14%	28%	19%	31%	7%	
(Presynch-Ovsynch)	4	48	95	64	104	24	Total 339

Genomics and its implementation in dairy cattle selection and management

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Introduction

In the past three years, tens of thousands of North American dairy cattle have been genotyped using the Illumina BovineSNP50 BeadChip, and alternative high-density and low-density genotyping chips have recently become available. These technologies became possible due to sequencing of the bovine genome and were developed via collaboration between Illumina Inc., the USDA Agricultural Research Service, the National Association of Animal Breeders, and other commercial and academic partners. A key breakthrough is the ability to carry out thousands of DNA marker tests simultaneously, for a cost of less than ½ ¢ per marker. Single nucleotide polymorphism (SNP) markers represent base changes (A, T, C, or G) within the DNA sequence of a cow or bull – a sequence that consists of approximately three billion base pairs distributed over 30 pairs of chromosomes. These SNP markers can be genotyped in an efficient and automated manner, in contrast to the labor-intensive genotyping methods that were used previously. Another key breakthrough is the finding that, once a large number of genetic markers become available for an individual animal, it is possible to estimate that animal's breeding value based on associations between marker genotypes and milk yield, somatic cell score, productive life, daughter pregnancy rate, and other key traits that were observed in other animals of the same breed. The most important animals in this process are the dairy bulls represented in the Cooperative Dairy DNA Repository, which was formed more than 15 years ago, when ABS Global, Accelerated Genetics, Alta Genetics, Genex Cooperative, Select Sires, Semex, and Taurus Service began storing semen samples from young bulls entering their progeny testing programs for the purpose of genetic research.

Keywords: Genetic improvement, dairy cattle, genomics, predicted transmitting ability, sire selection

Validation of genomic predictions by USDA

In a widely cited study by scientists at the USDA-ARS Beltsville Agricultural Research Center, a total of 5,369 Holstein bulls and cows that were born from 1952 to 1999 were genotyped with the Bovine SNP50 BeadChip.[1,2] Genotypes and phenotypes of these animals were used to estimate the effects of 38,416 SNP markers on production, type, longevity, udder health, and calving ability. Next, the estimated SNP effects were used to compute the genomic predicted transmitting abilities (PTAs) of 2,035 young Holstein bulls born from 2000 to 2003 that had no progeny of their own. Finally, the 2009 PTAs of bulls in the latter group, which were based on information from their progeny, were compared with their traditional parent averages and the genomic PTAs computed from 2004 data. The same process was repeated in Jerseys (using 1,361 older bulls and cows for prediction and 388 young bulls for validation) and Brown Swiss (using 512 older bulls and cows for prediction and 150 young bulls for validation). Results in Table 1 show the increase in reliability due to genomic information, as compared with the reliability from pedigree information only.

Table 1. Reliability changes due to the inclusion of genomic data in national genetic evaluations in the validation study of VanRaden et al.[1]

Trait	Increase in Reliability due to Genomics		
	Holstein	Jersey	Brown Swiss
Lifetime Net Merit	+24%	+8%	+9%
Milk Yield	+26%	+6%	+17%
Fat Yield	+32%	+11%	+10%
Protein Yield	+24%	+2%	+14%
Fat Percentage	+50%	+36%	+8%
Protein Percentage	+38%	+29%	+10%
Productive Life	+32%	+7%	+12%
Somatic Cell Score	+23%	+3%	+17%
Daughter Pregnancy Rate	+28%	+7%	+18%
Final Classification Score	+20%	+2%	+5%
Udder Depth	+37%	+20%	+8%
Foot Angle	+25%	+11%	-1%

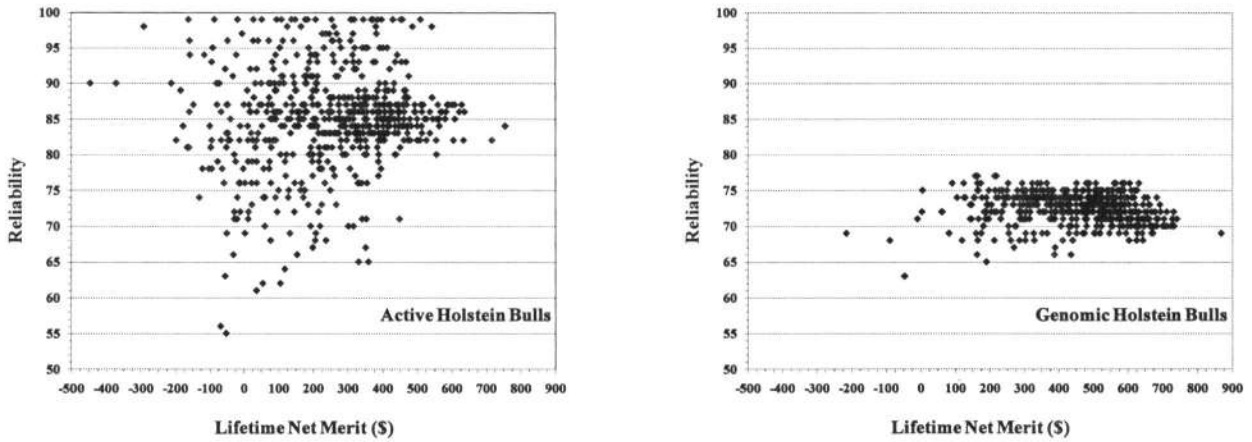
As shown in Table 1, gains in reliability from genomic information were significant for almost all traits and breeds, ranging from -1% for foot angle in Brown Swiss to +50% for fat percentage in Holsteins. Gains were largest for traits for which single genes with large effects had already been discovered, such as fat percentage (DGAT1 gene on chromosome 14)[3] and protein percentage (ABCG2 gene on chromosome 6).[4] For each trait, we can combine a young animal's pedigree with information regarding its SNP genotypes to obtain a genomic PTA of much greater accuracy. For a heifer calf, reliability of the genomic PTA is greater than the information we could obtain by measuring several lactation records on the animal and its daughters. For a young cow, genomic information can be combined with her lactation records to obtain a genomic PTA that is significantly more informative than her traditional PTA. For a bull calf, reliability of the genomic PTA is equivalent to what we could obtain by measuring performance on 25 or 30 of his progeny test daughters. Improvements in accuracy can even be obtained for bulls that have completed progeny testing, although the gain in information for a bull that already has performance data from 80 to 100 daughters is much smaller. Gains in reliability for Jerseys and Brown Swiss have not been as great as for Holsteins. However, this difference is largely due to the fact that fewer progeny tested bulls have been genotyped, and results for these breeds will be improved by combining information from North American sires with that of key populations internationally.

Impact on sire selection decisions

The artificial insemination (AI) studs are in the midst of tremendous change because of this technology. Virtually every young bull entering an AI company today is DNA tested on the farm and selected from a group of five to ten young bulls with similar pedigrees. Therefore, we know that each of these bulls has received a favorable sample of genes from its parents. The genomic PTA for a young bull typically has reliability in the range of 60 to 75%, as opposed to only 30 to 40% for its traditional parent average. North American AI companies are now marketing semen from hundreds of young bulls that have genomic PTAs but no daughters of their own. These young bulls have replaced older, proven bulls that were at the low end of the sire line-up, and many of these bulls are being used for contract matings. Because buyers now have the ability to distinguish between sets of full siblings that have the same parent average, the premium for securing first choice from a flush is much greater, and buyers at consignment sales and dispersals now pay a premium for young animals with favorable genotypes.

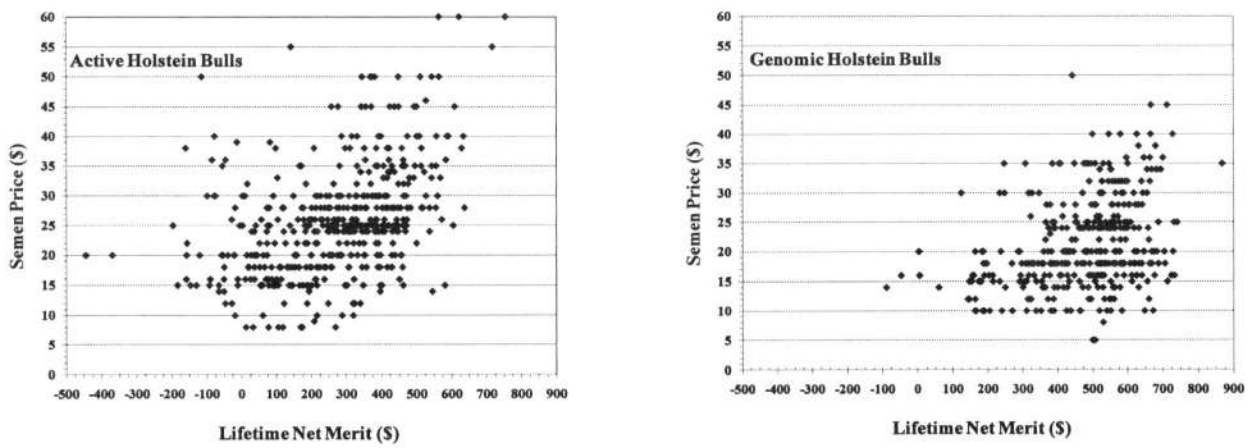
What about commercial producers? While these producers may not yet be genotyping young females on their farms, they are seeing semen on the market from hundreds of young bulls with genomic breeding values and no progeny. These bulls have attractive pedigrees, because they're younger than the current proven bulls, but their reliabilities are lower, as shown in Figure 1.

Figure 1. January 2011 PTA for Lifetime Net Merit versus reliability for Holstein bulls with active status based on progeny testing (left) and genomic status based on DNA testing (right).



Because reliabilities of young, genome-tested bulls are lower, producers should avoid heavy use of one or two top bulls and should spread out their risk by using a larger group of bulls. Avoiding these bulls entirely is a bad idea, even for risk-averse producers, because their genetic merit is high relative to their semen price, as shown in Figure 2.

Figure 2. January 2011 PTA for Lifetime Net Merit versus semen price for Holstein bulls with active status based on progeny testing (left) and genomic status based on DNA testing (right).



Development of inexpensive, low-density genotyping platforms

Because the cost of the BovineSNP50 BeadChip has largely limited its application to males and elite females, attention has focused on development of inexpensive alternatives that can capture the majority of the gain for a fraction of the price. Initially, we attempted to select the most important SNPs based on magnitude of their estimated effects.[5] Using August 2003 progeny test PTAs for Lifetime Net Merit of 3,305 Holstein bulls born from 1952 to 1998, we evaluated the ability of various subsets of SNPs to predict April 2008 progeny test PTAs for 1,398 Holstein bulls born from 1999 to 2002. Subsets were created by sorting the original 32,518 SNPs by the absolute values of their estimated effects and choosing the top 300, 500, 750, 1,000, 1,250, 1,500, or 2,000 SNPs. For reference purposes, subsets of 300, 500,

750, 1,000, 1,250, 1,500, or 2,000 equally spaced SNPs were also created. Correlations between these genomic predictions and corresponding PTAs from progeny testing are shown below, in Table 2.

Table 2. Correlations between progeny test PTAs for Lifetime Net Merit and genomic predictions from various subsets of SNPs in a population of 1,398 Holstein bulls, where SNPs were chosen based on spacing or size of estimated effect.[5]

Number of SNP Markers Genotyped	SNPs with Largest Effects	Equally Spaced SNPs
300	0.428	0.253
500	0.485	0.333
750	0.519	0.435
1,000	0.537	0.422
1,250	0.554	0.477
1,500	0.559	0.518
2,000	0.567	0.539
32,518	0.612	

The reference model with 32,518 SNPs provided a correlation of 0.612, whereas correlations between progeny test PTAs and genomic predictions derived from 300 to 2,000 selected SNPs ranged from 0.428 to 0.567. Correlations for sets of selected SNPs were consistently greater than for sets of equally spaced SNPs. In a related study, Vazquez et al noted that low-density chips containing SNPs with the largest estimated effects for Lifetime Net Merit would provide greater predictive ability for production traits than for fitness traits.[6] Furthermore, low-density assays composed of selected SNPs would be breed-specific and trait-specific. For these reasons, we determined that it would be more efficient to genotype a slightly larger set of equally spaced SNPs that would facilitate imputation of missing high-density genotypes, as suggested by Habier et al, rather than focus on a few hundred selected SNPs with large effects.[7]

To determine if imputation of high-density (i.e., BovineSNP50 BeadChip) genotypes from subsets of a few hundred or a few thousand equally spaced SNPs was feasible, we used a population of 2,656 Jersey bulls and 490 Jersey cows and heifers that had been genotyped for 43,385 SNPs. This population was divided into a reference panel, consisting of 2,542 animals born from 1953 to 2006, and a study sample, consisting of 604 animals born from 2007 to 2009. For animals in the study sample, genotypes were "masked" (i.e., hidden) for a randomly chosen 20, 60, 80, 90, 95, 98, or 99% of SNP markers. Three chromosomes were considered (BTA1, BTA15, and BTA28), but results are shown only for BTA15, which contained 1,377 SNP markers. After masking 20 to 99% of the SNPs, the number of SNPs available for imputing missing genotypes ranged from 14 to 1,102. Many algorithms have been developed for constructing haplotypes and imputing genotypes in humans, and in this study we used the method of Scheet and Stephens,[8] which was implemented via fastPHASE 1.2 software, and the method of Howie et al,[9] which was implemented via IMPUTE 2.0 software.

The proportion of masked SNP genotypes that were imputed correctly is shown in Table 3.

Table 3. Proportion of SNP genotypes that were imputed in a sample of 604 Jersey cattle, using a reference panel of 2,542 Jersey cattle, according to method of imputation and percentage of SNPs that were actually genotyped.[10]

Percentage of SNP Markers Genotyped	Method 1 (fastPHASE 1.2)	Method 2 (IMPUTE 2.0)
1%	0.701	0.730
2%	0.726	0.780
5%	0.780	0.890
10%	0.874	0.924
20%	0.951	0.932
40%	0.984	0.935
80%	0.992	0.930

The proportion imputed correctly ranged from 0.66 to 0.73 when only 1% or 2% of genotypes were unmasked in the study sample, versus 0.75 to 0.89 when 5 to 10% of genotypes were unmasked, as would be the case for a medium-density panel with 2,000 to 4,000 SNPs. This suggested that a low-density chip with approximately 3,000 equally spaced SNPs would be adequate for imputing high-density genotypes from reference animals of the same breed.

Next, we sought to determine the impact of imputing (more specifically, the impact of imputing errors) on the accuracy of genomic predictions for economically important traits in dairy cattle. Genotypes of 1,762 Jersey sires, with 42,552 SNP markers apiece, were used in conjunction with progeny test PTAs for milk yield, protein percentage, and daughter pregnancy rate. A group of 1,446 sires with ≥ 10 milking daughters in May 2006 were used as the reference panel, and the accuracy of genomic PTAs based on imputed genotypes was evaluated using 316 sires with 0 milking daughters in May 2006 and ≥ 10 milking daughters in April 2009. Next, we created equally spaced subsets in which all but 366, 741, 1,468, or 2,942 of the original SNP genotypes were masked. Masked genotypes were imputed using the method of Howie et al,[9] implemented via IMPUTE 2.0 software. After imputation, genomic predictions for milk yield, protein percentage, and daughter pregnancy rate were computed, and these were compared with the traditional PTAs of these bulls resulting from progeny testing. Results are shown in Table 4.

Table 4. Correlations between progeny test PTAs for milk yield, protein percentage, and daughter pregnancy rate and genomic predictions for these traits based on 366, 741, 1,468, 2,942, or 42,552 SNP markers, with imputation of missing genotypes, in a population of 316 Jersey bulls.[11]

Number of SNP Markers Genotyped	Milk Yield	Protein Percentage	Daughter Pregnancy Rate
366	0.367	0.468	0.470
741	0.525	0.546	0.572
1,468	0.649	0.676	0.619
2,942	0.673	0.740	0.642
42,552	0.673	0.770	0.674

As shown in Table 4, a low-density genotyping chip consisting of approximately 3,000 equally spaced SNPs (i.e., the so-called "3K chip") can provide genomic predictions for milk yield, protein percentage, and daughter pregnancy rate that are roughly 95% as accurate as predictions from the BovineSNP50 BeadChip, for a small fraction of the price.

Cost-effective strategies for genotyping females on commercial dairy farms

To investigate whether low-density genotyping of females on commercial dairy farms would be cost effective, and to determine the conditions under which a producer could maximize the benefits of this technology, a simulation study was carried out. We created 100 dairy herds, each comprised of 1,850 animals; these included 850 replacement heifers (450 heifer calves and 400 yearling heifers) and 1,000 milking cows (350 in first lactation, 250 in second lactation, 170 in third lactation, 120 in fourth lactation, 70 in fifth lactation, and 40 in sixth lactation). Each animal's genetic potential for Lifetime Net Merit was simulated, using an average of $45 and a standard deviation of $146; these values correspond to the current mean and standard deviation for sire-identified, milk-recorded Holsteins in the US national genetic evaluation system (http://aipl.arsusda.gov/eval/summary/pctl.cfm). Genetic improvement over time was taken into account by adjusting the average PTA by $26 per year, according to age of the animal. Reliability of genetic predictions varied, according to the availability (or lack thereof) of pedigree information, performance (milk-recording) data, and low-density (3K) DNA test results for a given animal, as shown below.

Table 5. Assumed reliability values for predictions of Lifetime Net Merit based on pedigree, performance, and low-density genotyping data ("Traditional" = no DNA testing, "Genomic" = DNA testing with 3K chip) for simulated animals in each age group.

Age Group	Ancestry Unknown		Sire-Identified		Full Pedigree	
	Traditional	Genomic	Traditional	Genomic	Traditional	Genomic
Heifer calves	0.00	0.50	0.20	0.57	0.34	0.67
Yearling Heifers	0.00	0.52	0.21	0.59	0.35	0.68
1st Lactation Cows	0.18	0.56	0.40	0.63	0.52	0.71
2nd Lactation Cows	0.22	0.59	0.44	0.66	0.55	0.73
3rd Lactation Cows	0.25	0.62	0.46	0.68	0.57	0.74
4th Lactation Cows	0.27	0.64	0.48	0.69	0.58	0.74
5th Lactation Cows	0.29	0.65	0.49	0.70	0.59	0.75
6th Lactation Cows	0.30	0.65	0.50	0.70	0.60	0.75

After generating true and estimated breeding values for these animals, where accuracy of the estimated breeding values varied according to age, extent of known ancestry, and presence or absence of genomic testing information, we carried out selection and culling decisions within in each herd. Producers selected the top 10, 20, 30, . . ., 90% of animals within each age group based on the aforementioned estimates of genetic merit, and the remaining animals were culled. Next, the average breeding value for Lifetime Net Merit of animals that were selected using pedigree plus genomic information was compared with that of animals that were selected from the same age group using pedigree information only. The average gain in genetic merit due to DNA testing was then compared with the cost of the test, which was assumed to be $35 per animal. This cost was prorated over the number of animals that were selected from a given age group, such that the break-even gain in breeding value was $350, 175, 117, 88, 70, 58, 50, 44, or 39 when the top 10, 20, 30, 40, 50, 60, 70, 80, or 90% of animals were selected, respectively. The fraction of genetic merit that was passed along to future generations was also considered, assuming that each female generated her own replacement, and that one-half, one-quarter, and one-eighth of her genetic superiority or inferiority would be passed along to her daughter, granddaughter, and great-granddaughter, respectively. When future generations were considered with a discount rate of 5% per year, the net present value of the break-even gain in breeding value was $206, 103, 69, 52, 41, 34, 29, 26, or 23, respectively, depending on the proportion of animals selected. Lastly, strategies were considered in which the producers pre-sorted animals based on pedigree information (if available) and then DNA tested the top 50% or bottom 50% of animals in each age group, rather than DNA testing the entire herd. Results are shown below.

Table 6. Average Lifetime Net Merit breeding values ($) for **heifer calves** selected based on genetic predictions from pedigree, performance, and low-density genotyping data (Trad = no DNA testing, All = DNA testing whole herd with 3K chip, Top = DNA testing top half of herd, Bot = DNA testing bottom half of herd) for simulated herds in this study. Cases in which testing costs are offset by gains in genetic merit in the current generation (underlined and bold) or in current plus future generations (underlined) are highlighted.

%	Unknown Ancestry				Sire-Identified				Full Pedigree			
Selected	Trad	All	Top	Bot	Trad	All	Top	Bot	Trad	All	Top	Bot
Top 10	245	**612**	**531**	389	474	628	630	503	550	664	667	563
Top 20	247	**537**	**443**	**361**	429	554	**540**	459	485	580	**577**	502
Top 30	245	**487**	**382**	**346**	395	501	**475**	427	444	523	**511**	462
Top 40	245	**445**	**344**	**334**	370	**458**	**419**	402	410	477	450	432
Top 50	246	**410**	**320**	**322**	350	**422**	381	382	381	436	404	405
Top 60	246	**378**	**302**	**312**	329	387	351	**364**	354	399	368	382
Top 70	246	**347**	**287**	**306**	311	355	324	**346**	329	364	338	**359**
Top 80	246	**318**	**274**	**296**	292	323	299	**320**	305	329	309	**329**
Top 90	246	**286**	261	**278**	272	289	275	289	279	293	280	293

As shown in Table 6, genomic testing of all heifer calves seems to be cost-effective if pedigree information is unavailable. This could be the case if replacements were purchased (or were about to be purchased) from a source that could not provide accompanying pedigree information, or if recording of ancestry had lapsed within a given herd. As expected, the value of genomic testing is lower in herds that routinely record sire identification, and lower yet in herds with several generations of pedigree data for every animal. Nonetheless, genomic testing of heifer calves may be cost-effective in such herds, particularly if animals are pre-sorted prior to testing.

Table 7. Average Lifetime Net Merit breeding values ($) for **first lactation cows** selected based on genetic predictions from pedigree, performance, and low-density genotyping data (Trad = no DNA testing, All = DNA testing whole herd with 3K chip, Top = DNA testing top half of herd, Bot = DNA testing bottom half of herd) for simulated herds in this study. Cases in which testing costs are offset by gains in genetic merit in the current generation (underlined and bold) or in current plus future generations (underlined) are highlighted.

%	Unknown Ancestry				Sire-Identified				Full Pedigree			
Selected	Trad	All	Top	Bot	Trad	All	Top	Bot	Trad	All	Top	Bot
Top 10	359	524	516	393	465	545	556	470	508	571	577	505
Top 20	317	447	**428**	353	403	466	469	411	435	484	489	437
Top 30	286	393	**366**	320	355	409	407	368	384	426	428	390
Top 40	262	**352**	**312**	296	317	365	349	333	343	379	369	352
Top 50	239	**315**	273	**275**	287	326	303	304	309	337	321	321
Top 60	220	**281**	242	**256**	259	290	267	279	277	299	282	293
Top 70	202	249	216	**237**	233	256	237	256	246	263	247	264
Top 80	184	217	193	**214**	206	221	208	224	215	227	215	229
Top 90	166	184	169	183	178	186	178	187	182	189	182	190

As shown in Table 7, the value of testing young cows depends heavily on the availability (or lack thereof) of pedigree data. One or two lactation records on a young cow cannot provide an accurate assessment of her genetic value if her ancestry is unknown, and in this case there is an opportunity to add significant accuracy through genomic testing. On the other hand, the amount of additional information

provided by genomic testing is relatively small for a pedigree-recorded cow that has lactation records of her own, and in this situation a producer should pre-sort the herd (perhaps more precisely than in this study, such as into thirds or quartiles) and test only those animals that are "on the bubble" with respect to a selection or culling decision. Although the value of genomic testing is greater if pedigree information is lacking, we do not advocate the use of genomic testing as a substitute for recording of ancestry. In fact, one cannot pre-sort the herd with any degree of accuracy without knowledge of each animal's sire, and preferably its maternal grandsire as well. Therefore, producers who keep accurate records of ancestry can more effectively target animals for DNA testing, and in this manner they can reap greater benefits from the technology.

Table 8. Average Lifetime Net Merit breeding values ($) for **fourth lactation cows** selected based on genetic predictions from pedigree, performance, and low-density genotyping data (Trad = no DNA testing, All = DNA testing whole herd with 3K chip, Top = DNA testing top half of herd, Bot = DNA testing bottom half of herd) for simulated herds in this study. Cases in which testing costs are offset by gains in genetic merit in the current generation (underlined and bold) or in current plus future generations (underlined) are highlighted.

%	Unknown Ancestry				Sire-Identified				Full Pedigree			
Selected	Trad	All	Top	Bot	Trad	All	Top	Bot	Trad	All	Top	Bot
Top 10	246	387	391	267	334	413	421	336	371	427	433	371
Top 20	197	311	**305**	215	266	324	332	267	293	336	341	293
Top 30	160	256	**236**	185	220	267	267	224	243	276	279	245
Top 40	130	211	**176**	158	182	220	211	189	199	225	220	205
Top 50	108	171	135	135	149	179	163	160	162	184	170	171
Top 60	84	134	100	**113**	118	142	122	135	126	146	131	143
Top 70	61	100	71	**93**	86	105	88	107	95	110	97	111
Top 80	38	66	44	**64**	56	70	57	71	63	73	62	74
Top 90	15	31	18	31	25	32	25	34	28	34	28	35

As shown in Table 8, the value of testing older cows within a herd is less than that of testing younger cows, and substantially less than that of testing yearling heifers and calves. Many animals have already culled themselves from the herd prior to fourth or fifth lactation, through poor performance, impaired health, or infertility, and therefore additional opportunities for culling are limited. Furthermore, using older animals to produce additional replacements, such as through embryo transfer or the use of gender-selected semen, will not be particularly beneficial, because these animals have fallen victim to genetic trend and are not genetically competitive with their daughters' and granddaughters' generations.

Conclusions

In summary, it is clear that genomic information can enhance the accuracy of genetic evaluations for bulls, cows, heifers, and calves. Breeding companies are now marketing hundreds of young bulls based solely on genomic information. These bulls have higher average genetic merit than older bulls that have completed progeny testing, but reliability values are lower. Using a single genome-tested bull very heavily is a significant risk, but ignoring these young bulls as a group has a heavy opportunity cost. To date, price has largely limited genotyping to males and elite females. However, the recent development of low-density assays that facilitate imputation of high-density genotypes from a reference population of AI sires allows users to capture the majority of benefits for a fraction of the price. This may lead to widespread adoption of genomic testing of cows, heifers, and calves on commercial farms, particularly in herds that lack pedigree information or herds that can effectively pre-sort animals based on pedigree data. Potential applications include selection among heifer calves or springing heifers on farms that have used gender-selected semen, screening of heifers or cows prior to purchase by herds that are expanding, evaluation of potentially elite heifers and cows that could provide added revenue through sale of breeding

stock, and eventually value-added services such as genome-enhanced mate selection and genome-guided management protocols.

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Management and economical considerations of timed artificial insemination and natural service breeding programs in dairy herds

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Introduction

Artificial insemination (AI) has proven to be a reliable technology for dairy producers to make genetic progress and control venereal diseases in their herds. However, despite these advantages many dairy producers prefer to use natural service (NS) as a component of their herd's breeding program.[1-5] Producers that use NS believe that more cows can be bred by NS than AI because human errors in estrus detection are avoided when bulls are used. However, several studies have shown that this perceived increased in estrus detection and thus more cows bred when bulls are used, does not result in better reproductive performance when compared to AI.[4,6] Furthermore, the development of breeding programs for AI at a predetermined time (timed AI; TAI), without the need for estrus detection has evolved in the last fifteen years as a successful strategy for reproductive management of lactating dairy cows.[7-9]

Dairy producers must consider that a successful reproductive program based on NS is dependent on fertile bulls that require proper management which can be labor intensive. In this manuscript, methods to manage NS breeding programs are discussed and research that compared reproductive performance and economics between TAI and NS breeding systems is reviewed.

Keywords: Artificial insemination, natural service, reproductive efficiency, reproductive management

Management considerations for natural service herds

Research on management strategies to optimize fertility in bulls used for NS in dairy herds is lacking.[10] Overton et al[11] reported that recommendations to use NS bulls in dairies are made from research conducted on beef bulls and from experiences working with dairy clients who successfully manage bulls. The ability of a bull to impregnate cows is dependent on semen quality, libido, mating ability and social ranking among other bulls and females.[12] Therefore, a breeding soundness evaluation (BSE), as recommended by the Society for Theriogenology,[13] is fundamental and only bulls classified as a potential satisfactory breeder should be used. A BSE should be performed in all bulls prior to cow exposure and should be repeated every six months to determine whether or not bulls maintain their reproductive soundness during cow exposure over time. As a component of the BSE, testing for the venereal diseases trichomoniasis and campylobacteriosis (vibriosis) is highly recommended. Young bulls (2 to 2.5 years) should be used because of their temperament and lower risk for venereal disease transmission.[14] Young bulls should have achieved full puberty and sexual maturity, which occurs around 14 months of age, and should not be undersized in relation to a mature Holstein cow.

Bulls should undergo the same vaccination (except for brucellosis and *Tritrichomonas foetus*) and parasite control practices as cows. Control of venereal diseases is essential to the success of NS breeding programs. Cows should be vaccinated for campylobacterosis at least three weeks prior to being exposed to bulls and receive a booster at intervals of six months. Bulls can also be vaccinated for campylobacterosis, with some success reported using twice the recommended dose for a cow.[14] Vaccination is also available for *Tritrichomonas foetus* in cows. The bull to open cow ratio (BCR) is an important management factor in herds that use NS. Champagne et al[1] reported that 53% of California dairymen surveyed used bulls at the ratio of 1 bull per 30 or fewer non-pregnant cows. The most common BCR reported was 1:20-25 total cows in the pen. Although, the optimal BCR for dairy herds has not been evaluated, housing type and environment are important considerations. For dry lot or pasture dairies, the BCR is most likely 1 to 20, but for free stall dairies more bulls are recommended and a BCR of 1:15-20 has been suggested.[11] Because safety should be a major concern with bulls on dairy farms, those bulls that exhibit a bad temperament should be culled. Other safety precautions include the use of younger bulls and strict adherence to safety protocols.

An example of a bull management program developed by Dairy Production Systems of Florida (High Springs, Florida; http://dpsdairy.com) is shown below with their permission.

All new bulls:

All purchased bulls should be mouthed for age. Bulls older than 18 months of age should be rejected. All bulls must weigh 700-800 lb at the time of purchase and each bull should have its own unique identification number.

- Perform a BSE, test for trichomoniasis and test for PI BVD by ear notch method.
- Vaccinations:
    i. IBR/BVD/PI3 & BRSV (modified live vaccine) + 5-way Lepto and L. borgpetersenii. Repeat initial vaccination in 3 weeks.
    ii. Clostridium 8-way
    iii. Campylobacter (oil adjuvant): Revaccinate with campylobacter vaccine every 3 months.
- Parasite control:
- Deworm and delouse: Repeat 3 weeks after first application

Current breeding bulls (exposed to lactating cows)

- All bulls must have a complete BSE every 6 months. After initial processing and clearance, bulls should be used for 6 months. After 6 months bulls should be re-tested and if satisfactory, they are used for another 6 months, after which the bull is culled.
- No bull is to be used in service for more than 12 months.
- Bulls receive BSE, trichomoniasis test, re-vaccination for campylobacter each 6 months. Other vaccines are boostered in concert with the lactating herd.
- Bulls must be checked daily for lameness and any other health disorders. If a bull is lame he should be removed from the herd and treated accordingly and replaced immediately by a sound bull.
- Keep a minimum of 10 bulls in the resting pen ready to relieve any ill or lame bull. (These additional "bulls-in-reserve" represent about 10 % of the normal working population.)
- Monitor attitude daily. Any bull that becomes aggressive or difficult to handle must be culled immediately.
- Check daily to make sure that bulls are in the correct pens and that bull-to-cow ratios are correct. Bulls should be rotated and rested after 14 d. Maintain 1 bull for every 20 open cows in each pen. After each palpation week, re-evaluate these ratios and adjust accordingly.
- Resting bulls receive the lactating cow TMR refusals (tends to be higher in fiber and contains less cottonseed and energy as the original feed, but yet decreases the risks associated with wholesale ration changes)

A NS breeding program allows for the implementation of important management practices such as a postpartum herd without bull presence with a designated voluntary waiting period. A postpartum herd allows for cows to be monitored daily for health, and sick cows treated promptly without the nuisance of having a bull present. The postpartum herd also allows a well-balanced transition diet to be fed to help control metabolic or digestive disorders. Furthermore, prostaglandin $F_2\alpha$ (PGF) can be administered to cows prior to being exposed to bulls to help synchronize estrual events.

In cows bred by NS, accurate estimation of gestation length may be difficult and results in cows not receiving an appropriate dry period which can affect cow performance after calving. The length of the dry period was associated with udder health, culling, and overall performance during early lactation.[15] Extended dry periods of 143 to 250 days increased the likelihood of subclinical mastitis during early

lactation and had a negative impact on reproductive performance. Short (0 to 30 d) and extended (90 days) dry periods had a detrimental impact on early lactation and 305 day milk yield and increased the risk of overall culling when compared to a conventional dry period of 53 to 76 days. Estimates of days pregnant obtained from palpation are reliable from 32 to 90 d. Assuming that a pregnancy diagnosis of 32 days is the earliest that can be performed, the interval between examinations of non-pregnant cows should not be greater than 60 days. In this manner, a cow that is less than 32 d pregnant and is diagnosed non-pregnant would be between 61 to 91 days pregnant when re-confirmed 60 days later. The date of the last examination at which the cow was diagnosed not pregnant is important information for estimating gestation length. Cows that are found to be cystic can be treated with gonadotropin-releasing hormone (GnRH); use of PGF should be limited only to cows with pyometra. To monitor the presence of trichomoniasis in a herd, some practitioners have found it beneficial to re-confirm pregnant cows between 90 to 120 d of gestation.[16] Abortions due to *Tritrichomonas foetus* occur during the first trimester of gestation and rarely after five months of gestation.[17] Pyometra may be present in up to 10 percent of the cows in an outbreak of trichomoniasis.[17] Therefore, it is strongly recommended that during routine reproductive examination, cows diagnosed with pyometra should be cultured for *Tritrichomonas foetus*. Trichomonad pyometra is post-coital and not postpartum and occurs after death of the developing embryo or early fetus.[17] Pregnancy in cows should also be re-confirmed prior to dry off similar to the practice used in AI herds.

Reproductive performance of timed ai vs. natural service

Natural service and TAI are two breeding programs that can be used by dairy producers to avoid detection of estrus to breed cows by AI. Lima et al[18] compared reproductive performance between NS and TAI bred lactating dairy cows. Cows were randomly allocated to a NS or TAI group. Cows in both groups were presynchronized with two injections of PGF given 14 days a part. Fourteen days after the last PGF injection, cows in the TAI group were enrolled in an Ovsynch program (d 0 GnRH; 7 d later, PGF; 56 h after PGF injection, second dose of GnRH; and 16 h after second GnRH cows were TAI). All cows in the TAI group received an intravaginal device containing progesterone inserted 18 days after TAI and GnRH on day 25 after AI as part of an aggressive strategy to resynchronize cows shortly after pregnancy diagnosis. Cows were examined by ultrasonography on day 32 after TAI; non-pregnant cows received PGF and GnRH 56 h later followed by TAI 16 h after the GnRH injection. Non-pregnant cows in the TAI group were re-synchronized and re-inseminated up to five times using the same program.

Cows in the NS group were exposed to bulls 14 days after the second PGF injection, and ultrasonography was performed on day 42 after exposure to bulls to determine pregnancy status. Non-pregnant cows in the NS group were reexamined by transrectal palpation combined with ultrasound every 28 days until diagnosed pregnant or 223 days postpartum, or whichever occurred first. Cows diagnosed pregnant in TAI or NS were re-examined 28 days later to determine pregnancy loss.

Bulls underwent a BSE and entered the NS program if classified as a satisfactory potential breeder according to the guidelines of the Society for Theriogenology.[13] Breeding soundness evaluations were repeated every three months and bulls that graded unsatisfactory were replaced. The BCR was one bull per twenty cows, the ratio in each pen was maintained based on the number of cows diagnosed non-pregnant. Bulls were rested for 14 days after 14 days of cow exposure, were vaccinated according to farm standard operating procedures and removed from the herd after 12 months of use. Blood was collected from cows and analyzed for progesterone to determine cyclic status and body condition scored at day 70 postpartum.

The overall 21-day cycle pregnancy rate was not different between groups (25.7 and 25.0% for NS and TAI, respectively). The adjusted hazard ratio (AHR) for pregnancy was greater for NS than TAI (Figure), which resulted in fewer median days open (111 vs. 116 days). Proportion of pregnant cows at 223 days postpartum was greater in the NS than TAI group (84.2 vs. 74.8%, respectively). Cyclicity did not affect reproductive responses. Cows with a body condition score ≥2.75 had a greater proportion of pregnant cows in the first 21 days of breeding and AHR for pregnancy in the first 223 days postpartum.

Primiparous cows had greater proportion of pregnant cows and AHR than multiparous cows at 223 days postpartum.

The dynamics of the NS reproductive program allowed all eligible cows to have a breeding opportunity every 21 days which allowed cows in this group to have up to eight breeding opportunities until 223 days postpartum which was the endpoint for the study. On the other hand, cows enrolled in the TAI program due to the strategy of only TAI, breeding opportunities only occurred every 35 days, allowing for a maximum of five breeding opportunities until the end of the study at 223 days postpartum. Therefore, the greater proportion of pregnant cows in the NS group was a result of increased number of opportunities for breeding occurring in this program in comparison to the TAI group. Nonetheless, the most common measurement of reproductive performance, the 21-d cycle pregnancy rate was not different.

A second study was conducted in Florida[19] in which all cows were TAI for the first service and then either were exposed to NS one week later or were TAI up to three times before being moved to a NS group. In this study, all cows received a double Ovsynch TAI program (d -27 GnRH, d -20 PGF, d -17 GnRH, d -10 GnRH, d -3 PGF, d -1 GnRH, and d 0 AI) for first AI. On the day of first AI, cows were blocked by parity and randomly assigned to receive one (1TAI) or 3 TAI (3TAI) before being moved to a NS pen. Cows in the 1TAI treatment were moved to NS seven d after the first AI and cows in the 3TAI treatment seven d after the third TAI. Pregnancy status was determined 32 days after TAI and every 28 days in the NS herds after the previous non-pregnant diagnosis.

Non-pregnant cows in the 3TAI group were resynchronized with the Ovsynch program starting on day 32 after the previous insemination, such that the re-insemination interval was 42 days. Pregnant cows were re-evaluated for pregnancy 28 days after the initial diagnosis. Cows were scored for body condition 32 days after the first AI. All cows had a period of 231 days after the first AI As expected, pregnancy at the first TAI did not differ between 1TAI and 3TAI on day 60 after insemination (3TAI=33.4 % vs 1TAI=31.5 %). Cows receiving 3TAI had 15% greater AHR for pregnancy than 1TAI. This resulted in smaller median days open for 3TAI than for 1TAI (3TAI=123 d vs 1TAI=143 d). The proportion of pregnant cows in the first 21 days was not different (3TAI=33.6 % and 1TAI=32.6 %). Nonetheless, the proportion of pregnant cows in the 42 days was greater for 3TAI than for 1TAI (3TAI=51.4 % vs 1TAI=41.5 %). The 21-day cycle pregnancy rate was greater for 3TAI than 1 TAI (3TAI=26.7 % vs 1TAI=23.6%). Therefore, in spite of the long re-insemination interval, cows receiving 3TAI had improved reproductive performance than those receiving 1TAI and was attributed to the 10% increased proportion of pregnant cows in the first 42 days generated by the successful second TAI.

The results of these two studies indicate that TAI despite long re-insemination intervals either did not compromise or enhanced reproductive performance when compared to NS proving to be a successful alternative to eliminate the issues with estrus detection and the disadvantages of NS.

Economic considerations of TAI and NS

A study conducted in Florida, modeled potential net returns per cow by comparing use of TAI in winter and summer compared to insemination at detected estrus.[20] The greatest impacts on net returns were obtained when TAI was used during summer compared to winter. This finding was attributed to lower estrus detection rates observed during the summer months. It was concluded that use of a TAI program such as OvSynch is an economical alternative in reproductive management of dairy herds with poor estrus detection.

A TAI program using OvSynch was compared to AI at detected estrus in two large dairy herds differing in reproductive management.[21] Use of OvSynch reduced intervals to first AI and days open in both herds, as well as culling for infertility in herd 2. Conception rates for first AI at detected estrus were significantly higher compared to TAI in both herds and for overall inseminations at estrus in herd 2. For groups assigned to AI at estrus, mean 21-day submission rates over 200 d for AI were higher in herd 1 than in herd 2 (55.6 vs 28.6%). Days open and culling were the major cost factors. Although OvSynch improved reproduction in both herds, AI based on detected estrus was economically superior in herd 1, whereas OvSynch was superior in herd 2. The authors concluded that evaluation of synchrony protocols

should consider reproductive performance along with costs associated with treatments. Such costs may offset benefits to reproduction in herds with good estrus detection rates.

A direct comparison of the economics of TAI and NS was performed using as input the similar reproductive outcomes from the field trial[18] that compared these two breeding programs.[22] A herd budget including all costs and revenues was created taking into consideration all inputs relevant to determine the precise cost of each reproductive program. Net cost during the field study for the NS program was $100.49/cow per year and for the TAI program was $67.80/cow per year, unadjusted for differences in voluntary waiting period for first insemination (VWP) and pregnancy rates (PR). After inclusion of the differences in VWP and PR, the economic advantage of the TAI program was $9.73/cow per year. The costs per day per cow eligible for insemination were estimated at $1.45 for the NS program and $1.06 for the TAI program. Sensitivity analysis revealed that if the marginal feed cost increased to $5/hundredweight, which resembles the most common marginal feeding cost for most dairies, the advantage of TAI increased to $48.32/cow per year. If a marginal feed cost of $8/hundredweight was used then the profit from TAI in comparison to NS was more than tw0 times greater ($109.58/cow per year). In addition, higher milk prices and greater genetic progress increased the advantage of TAI as well. Nonetheless, if dairy producers opt to use semen of $22 instead $6, keeping the other input unchanged, the NS program had an economic advantage. If each NS bull present at lactating herd was replaced by an additional cow, the advantage of the TAI program was also greater ($60.81/cow per year). Setting the PR for both programs at 18% and the VWP at 80 d resulted in an advantage of $37.87/cow per year for the TAI program. In summary, this study showed that TAI was cheaper than NS in most of the scenarios evaluated and the reason for this advantage was depended greatly on cost to feed bulls, milk price, genetic merit and the consideration of replacing or not bulls in the lactating pen by cows.

Conclusion

Timed AI and NS are two successful alternatives that dairy producers can use to eliminate estrus detection to breed lactating dairy cows. Both programs can achieve acceptable reproductive performance. It is critical to emphasize to dairy producers that dairy bulls can be dangerous and require very strict management to produce acceptable pregnancy rates. Timed AI is less expensive than NS and therefore it's application is a better choice to eliminate problems of estrus detection in reproductive programs for lactating dairy cows.

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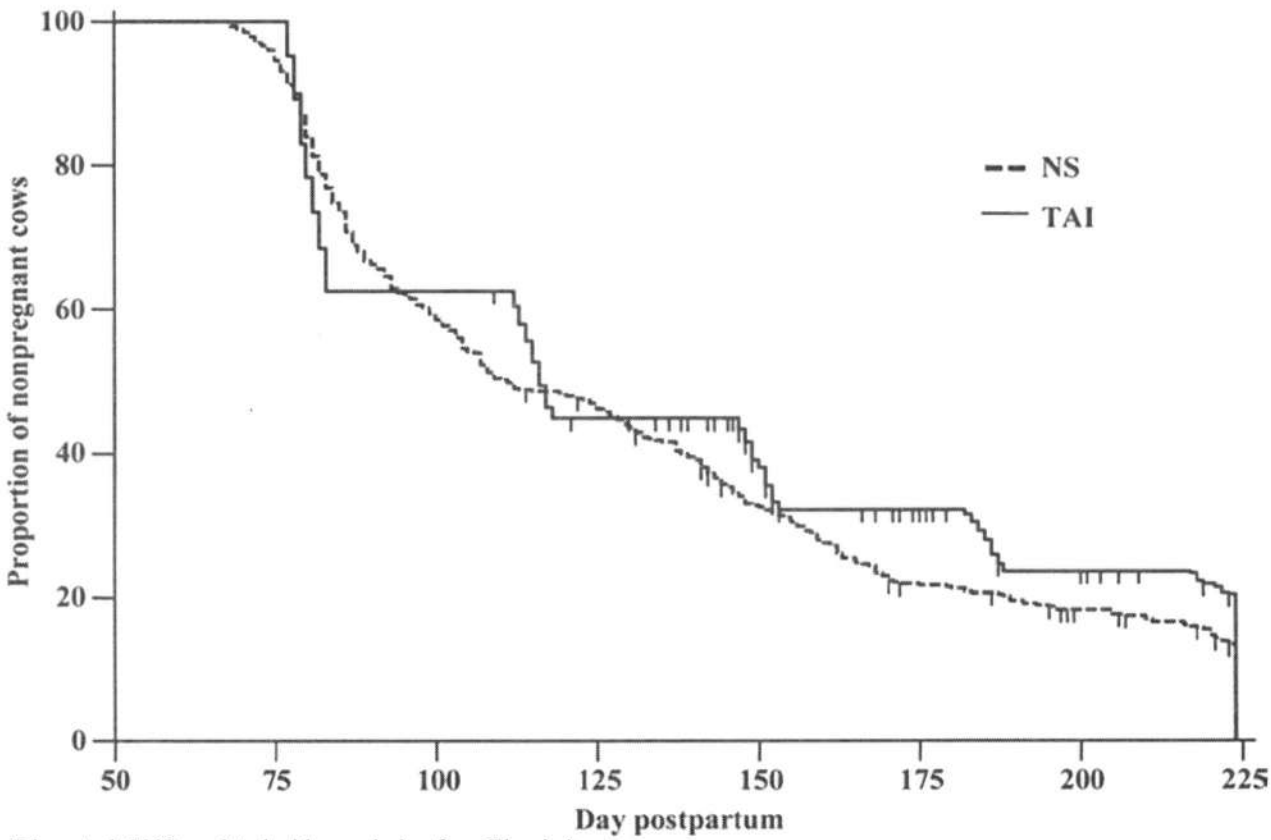
Figure. Survival curves for proportion of non-pregnant cows by days postpartum for cows bred by natural service (NS) or timed AI (TAI) in the first 223 d postpartum. Median interval to pregnancy for NS and TAI groups was 111 d (95% confidence interval [CI]=104 to 125) and 116 d (95% CI=115 to 117), respectively. The rate of pregnancy in the 223 d postpartum was greater (P=0.05) for NS than TAI (adjusted hazard ratio=1.15; 95% CI=1.00 to 1.31).



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Problems of the accessory sex glands

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Abstract

Problems of the accessory sex glands are reported in stallions. Seminal vesiculitis, although uncommon, is nonetheless the most frequently reported inflammatory/infectious pathology of the upper reproductive tract in stallions. Sperm occlusion of and accumulation within the ampullae is a common problem in breeding stallions, particularly following periods of sexual rest. It is important for theriogenologists to recognize these problems of the ampullae as they are typically highly treatable. In this presentation, the diagnosis and treatment of seminal vesiculitis will be reviewed. Sperm occlusion of and sperm accumulation within the ampullae also will be reviewed and data from a new case series will be presented. Finally, a case series of prostatic masses in geldings will be introduced.

Keywords: Stallion, accessory glands, seminal vesicles, ampullae, prostate

Introduction

The normal stallion possesses a full complement of accessory sex glands, including paired ampullae, paired seminal vesicles, a single, bilobed prostate, and paired bulbourethral glands. These glands function in adding fluid volume, enzymes, amino acids and buffers to the ejaculate.[1,2] The paired ampullae are the most cranial glands and are thickenings of the ducti deferentia proximal to the entry of the ducts into the urethra. Just caudal and lateral to the ampullae are the paired seminal vesicles. The seminal vesicles extend laterally from the midline at an approximate 40-degree angle to the urethra. When empty, the seminal vesicles become flattened and when distended with fluid they become roughly oval.[3] The seminal vesicles empty into the urethra with the ampullae at the seminal colliculus. The isthmus of the prostate is located on the ventral midline caudal to the seminal vesicles and dorsal to the trigone region of the bladder. The right and left lobes of the prostate extend laterally from the isthmus. The prostate gland empties into the urethra through numerous prostatic ducts. The paired bulbourethral glands are roughly spherical and are located on either side of the urethra just cranial to the anal sphincter at the ischial arch.

Seminal vesiculitis is reported infrequently, but can have a significant impact on reproductive function. Additionally, although ampullary blockage and sperm accumulation in the ampullae are frequently discussed in the clinical setting and have been reviewed in several texts, only one case series describing blockage of the ampullae has been reported.[4] Prostatic disease and clinically significant problems of the bulbourethral glands have not been reported in the horse to our knowledge. This presentation will describe the clinical features of seminal vesiculitis, and will provide additional detailed descriptions of the features of sperm accumulation and ampullary blockage in a new case series of stallions. Additionally, the clinical signs and progression of recently observed prostatic masses in geldings will be introduced.

Seminal vesiculitis

Although uncommon, seminal vesiculitis is the most frequently reported inflammatory/infectious pathology of the upper reproductive tract in stallions.[5-11] Affected animals typically are presented for gross abnormalities of the ejaculate (hemospermia, pyospermia, discolored semen, and/or clumps of debris in the ejaculate) poor semen quality (most often due to reduced longevity of sperm motility) and/or subfertility. In one instance, signs of colic were attributed to seminal vesiculitis and we have similarly observed a stallion with pain-related self-mutilation that appeared at least in part to be associated with severe seminal vesiculitis.[9,12] Affected stallions also may experience pain during ejaculation and thus may be presented for ejaculation failure. Microscopic examination of ejaculated semen may reveal all or any of the following: numerous neutrophils, bacteria (sometimes intracellular), red blood cells, and reduced sperm motility or reduced longevity of sperm motility. These findings should prompt further

examination of the stallion's genitalia to determine the source of the problem. Palpation per rectum of affected glands may reveal no abnormalities. However, in some acute cases, the affected gland is enlarged, painful, and filled with fluid.

The ultrasonographic appearance of affected seminal vesicles may aid in the diagnosis of seminal vesiculitis, although it is not always diagnostic.[5,13] Ultrasonographically, fluid within the affected gland(s) can vary from anechoic to relatively echogenic and often will contain particulate debris or fibrin tags. The lumen of the gland may be irregular. Familiarity with the appearance of normal seminal vesicles is very helpful since normal glands in sexually stimulated stallions also can become dramatically enlarged and filled with anechoic fluid. In cases of chronic seminal vesiculitis, affected glands become firm to hard and may contain little or no fluid.

Beta-hemolytic streptococcus, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus* spp., *Brucella abortus*, *Acinetobacter calcoaceticus* and *Streptococcus equisimilis* have been reported as causative agents of seminal vesiculitis in stallions.[8,10,14-16] The causative organism often can be isolated through bacteriologic culture of ejaculated semen, although additional cultures, including cultures of the penile urethra before and after ejaculation and of the external surface of the penis also typically are indicated. Fractionation of the ejaculate and culture and cytologic examination of each fraction may aid in diagnosis since the contents of the seminal vesicles should be expelled predominantly or exclusively in the final fractions.[17] Culture results must be interpreted judiciously since it is not uncommon to isolate contaminant bacteria originating from the penis or even the artificial vagina. A pure, heavy growth of an organism together with neutrophils in the ejaculate is likely to be significant.

Appropriate systemic antimicrobial therapy based on sensitivity results can then be instituted. However, systemic antimicrobials alone may not be curative, even if a prolonged and often expensive course of therapy is undertaken.[9] Alternatively, or in addition to systemic antimicrobials, a 1 m pediatric flexible endoscope can be passed through the urethra, and a catheter or culture swab can then be advanced directly into the affected gland via the seminal colliculus to aspirate and/or culture its contents.[18] In some cases, the endoscope itself can be advanced through the seminal colliculus directly into each seminal vesicle. This permits visualization of the lumen of the vesicles and their contents. This approach can also aid in treatment. A small plastic catheter can be advanced into the gland through the biopsy channel of the endoscope and the gland can be lavaged with sterile saline and then infused with appropriate antimicrobials. We treat affected stallions once daily using this technique and treatment is continued as indicated (generally one to two weeks). In the author's experience repeated cannulation of the seminal colliculus, as is often done for daily treatment, can lead to inflammation of the entrance to the glands and make future attempts at cannulation more and more challenging. Irritating antimicrobial infusions can potentiate inflammation. Progressive inflammation can obscure the opening of each colliculus and also can mechanically reduce the size of the openings.

It is our observation that one or more semen collections prior to intravesicular lavage and infusion help to evacuate the glands prior to antimicrobial infusion, and so may facilitate successful therapy. Systemic nonsteroidal anti-inflammatory drugs also can be included in the treatment plan. Evaluation of semen quality and regular ultrasonographic evaluations of affected glands can be used to monitor response to treatment.

One report of seminal vesiculectomy in a stallion has been published. Surgery was performed via a perineal incision and the affected seminal vesicle was removed with an emasculator in a manner similar to the technique used in bulls. In this case, surgery was curative.[10] Alternative surgical techniques have also been reported.[16,18] Note that seminal vesiculectomy may result in a decrease in the percentage of morphologically normal sperm.[19,20]

In our experience, the prognosis for stallions with seminal vesiculitis is fair. Some cases reportedly resolve spontaneously while others remain refractory despite extensive therapy. Anecdotally, we have had good results in stallions treated with intravesicular antimicrobials combined with nonsteroidal anti-inflammatory drugs, although the cost of treatment is high and in some cases the problem recurs.

Prostatic masses

Over the past four years, we have examined four geldings that were presented for dysurea associated with prostatic masses. Masses were identified on palpation and ultrasonographic examination per rectum. In case one, portions of the mass extended into the urethra via the prostatic ducts. Surgical debulking of the mass was performed endoscopically through the urethra and resulted in a temporary improvement of clinical signs. Signs recurred within several months in association with regrowth of the mass and the horse was euthanized. Prostatic cystadenoma was confirmed postmortem. In case two, palliative treatment was unsuccessful and the horse was euthanized within nine months of diagnosis. Postmortem examination confirmed the presence of a leiomyosarcoma involving the prostate. Case three was euthanized within one year of diagnosis after unsuccessful palliative treatment, but was not available for postmortem examination. In the fourth case, a concurrent urolith was identified and removed surgically. Clinical signs abated and this animal is doing well approximately nine months postoperatively in spite of persistence of the prostatic mass.

Prostatic masses should be considered as differentials in male horses presented for dysuria. The prognosis for affected animals appears guarded. However, in some cases prostatic masses may be incidental findings and it is possible that these masses occur more frequently than is apparent but remain undiagnosed in the absence of clinical signs. Treatment options for prostatic masses in horses are limited because of the difficulty of obtaining prostatic tissue via biopsy and the surgical inaccessibility of the gland.

Sperm occlusion of and sperm accumulation within the ampullae

Sperm occlusion of the ampullae was first described in a group of six stallions in 1992.[4] The condition is believed to result from sperm accumulating within the crypts and lumenae of the ampullae as the glands narrow prior to entry into the seminal colliculus. Over time, if the stallion is at sexual rest, these accumulations can continue to grow and eventually occlude the gland. Since the lumenae of the ampullae are continuations of the ducti deferentia, occlusion blocks passage of sperm from the testicles and epididymides and results in the clinical presentation of subfertility or infertility associated with oligospermia or azoospermia. It has been suggested that stallions with large testicles may be predisposed to this condition due to the associated higher sperm production.[16] Stallions with bilaterally occluded ampullae present for infertility associated with azoospermia. Alkaline phosphatase levels in these ejaculates are low since the ejaculate contains no contributions from the testes or epididymides.[21,22] In contrast, stallions with testicular origin azoospermia will have higher levels of alkaline phosphatase in the ejaculate, thus aiding in differentiating between the two conditions.

If the blockage becomes completely or partially dislodged, variable numbers of sperm will appear in the ejaculate. These sperm typically are damaged due to prolonged exposure to body temperature. Thus, asthenozoospermia and teratospermia are common, classically in association with a high percentage of tailless heads. In all of the six cases previously reported, palpable and ultrasonographic abnormalities of one or both ampullae were reported including ampullary enlargement, changes in ampullary tone, and changes in echogenicity.[4]

Treatment was based largely on a combination of ampullary massage per rectum and frequent semen collections with the goal of breaking up and clearing accumulated sperm. Oxytocin (20 IU iv immediately prior to semen collection) is often used to promote smooth muscle contractions. We have used substantially higher doses (up to 60 IU) with no obvious untoward effects. In protracted cases, 25–125 μg of cloprostenol can be administered intramuscularly approximately five minutes prior to semen collection.[16] It has been suggested that intractable cases may benefit from antegrade catheterization and flushing of the ductus deferens near the tail of the epididymis.[16,23]

During treatment, as the occlusion is breaking up, sperm numbers tend to be highly variable and, during or following dissolution of the occlusion, sperm numbers often increased beyond what would be expected for testicular size. Stallions with only one functional testis appeared to be at increased risk, probably because these animals would be rendered azoospermic by even a unilateral ampullary blockage, assuming that the blockage was ipsilateral to the functional testis.[4]

We have examined information from a second group of stallions diagnosed with sperm occlusion of the ampullae and, in concurrence with the findings of Love et al[4] have found no age or apparent breed predisposition (age range 3–30 years; numerous breeds represented). A disproportionate number of stallions were presented between February and March following prolonged sexual rest experienced during the nonbreeding season. Most stallions had ultrasonographic abnormalities of the ampullae, including hyperchoic material in the ampullary lumenae, distended ampullary lumenae, and/or heterogeneous glandular parenchyma. However, a minority of stallions had no obvious abnormalities on ultrasound. Thus, ultrasonography alone should not be used to rule in or rule out this condition.

Over half of the affected stallions had one abnormal or missing testis (hemicastration, unilateral cryptorchid, testicular tumor, unilateral degeneration), confirming that these animals are at increased risk and suggesting that unilateral blockages of the ampullae may be under-diagnosed in stallions with two normal testes. In stallions with unilateral blockages in which the contralateral testis is cryptorchid or diseased, alkaline phosphatase in the ejaculate remained elevated, thus complicating the diagnosis.

Treatment in all cases was built around frequent semen collections or frequent natural breedings. Oxytocin and/or prostaglandin and ampullary massage were administered to most, but not all stallions and the blockage resolved regardless. Time to resolution varied from one to ten days.

A potentially related condition, often called sperm accumulation syndrome, also is reported as a cause of subfertility in stallions. This condition has not been well-described in the reviewed literature, but has been discussed clinically and reported in texts as a variation on sperm occlusion.[16,24] In this syndrome, it is hypothesized that sperm accumulate within the ampullae in association with sexual rest. However, the accumulation does not fully block the lumen of the gland. Thus, sperm continue to appear in the ejaculate, although sperm numbers may be highly variable. Like sperm occlusion, most sperm that reach the ejaculate are damaged probably due to prolonged exposure to body temperature within the ampullae. Thus, stallions affected with sperm accumulation also present with poor semen quality for testicular size and character, particularly when the stallion is at sexual rest or is breeding on only a very limited basis. The poor semen quality is typically due to both poor sperm motility and poor sperm morphology often, but not always, in association with a high percentage of tailless heads.

A necessary component of the stallion's clinical picture is that semen quality improves to a variable degree when the stallion ejaculates frequently, presumably because accumulated sperm are being flushed out over time, thus allowing for a higher percentage of 'fresh' sperm from the testes and epididymides to reach the ejaculate. This 'definition' of sperm accumulation has not been standardized and, probably as a result, methods of diagnosing and treating the condition vary considerably. Depending on how rigid one sets the standards for diagnosis (e.g. it could be argued that any stallion showing improvements in semen quality with frequent collections could be affected by sperm accumulation syndrome), the incidence of the condition has been suggested to be as high as 30–40% following extended periods of sexual rest.[16] In this presentation, the clinical findings in a series of stallions presenting for evaluation and treatment of sperm accumulation syndrome will be discussed. Specific case histories will be used to illustrate the characteristics of these problems.

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Processing techniques for cooled shipment of stallion semen*

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Abstract

Application of sound semen-processing techniques can have a profound impact on semen quality of stallions following cooled transport that can translate to improved fertility. Use of high-quality semen extenders, appropriate extender-antibiotic types and concentrations, sufficient extender:semen dilution ratios, and proper cooling rates and storage temperatures are important to maximizing semen quality following cooled storage. Further enhancements in semen quality following cooled transport can oftentimes be gained by cushioned centrifugation of semen and resuspension of resulting sperm pellets in extender. In selected circumstances, centrifugation of sperm through a density gradient can lead to improved quality of the recovered sperm population. This procedure can also be applied to effectively remove seminal plasma from semen in instances where the seminal plasma of a stallion is known to be deleterious to semen quality. This communication provides an assortment of management techniques used with specific clinical cases to optimize semen quality prior to insemination or cooled storage.

Keywords: Stallion, cooled semen, breeding management, cushioned centrifugation, gradient centrifugation, low-dose insemination

Introduction

Cooled transport of extended semen has become commonplace in the equine breeding industry. Many stallions that yield high rates of fertility with fresh semen also have commercially acceptable fertility with cool-transported semen following simple dilution in high-quality extenders. Causes of reduced fertility with cool-transported semen can be multifactorial, and can range from improper reproductive management of mares, to specific issues of mare subfertility, to intrinsic stallion factors that negatively impact fertility, to mismanagement of stallion semen for cooled transport. This communication addresses methods to optimize semen quality and fertility of stallion semen. Emphasis is placed on methods for cushioned centrifugation of semen and for density-gradient centrifugation of semen. Clinical-case management is used to demonstrate the effectiveness of these procedures.

General considerations

The literature is replete with information relating to the effects of extender composition, semen:extender dilution ratio, and cooling rate/storage temperature on semen quality following cooled storage. These topics are beyond the scope of this communication, but excellent reviews are available.[1-6] The concentration of seminal plasma in extended semen can have a profound impact on semen quality following cooled storage.[7] As such, a general rule in our laboratory is to dilute semen with extender such that seminal plasma concentration is $\leq 0\%$ (v/v) following simple dilution. As some seminal plasma appears to be necessary for maximizing sperm survival when conventional extenders are used,[8] a minimum of 5% (v/v) seminal plasma is retained in extended semen in our laboratory. Given these criteria, the semen:extender ratio is 1:4 to 1:20 under usual circumstances. An added criterion in our laboratory is that final sperm concentration remains $> 25 \times 10^6$ per mL following dilution of semen with extender. To meet these conditions for minimizing seminal plasma and maintaining adequate sperm concentration in extended semen following simple dilution, the sperm concentration in raw (neat) semen

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must be $\geq 125 \times 10^6$/mL. Otherwise, centrifugation of extended semen and resuspension of sperm pellets in extender is required to reduce seminal plasma concentration while maintaining adequate sperm concentration. Provided seminal plasma concentration in extended semen is reduced sufficiently following this procedure, the concentration of sperm in the resulting extended-semen sample can be as high as $100–250 \times 10^6$/mL, without negatively impacting semen quality.[9,10] Under clinical conditions, the sperm concentration in the resuspended sample can be as high as 400×10^6/mL with no untoward effects on semen quality, in comparison with more dilute samples (D. Varner, personal observations).

In raw ejaculates with dilute sperm concentrations or in circumstances wherein seminal plasma must be removed because of apparent toxic effects on semen, centrifugation of semen must be performed. Under such situations, cushioned centrifugation of semen is becoming commonplace because sperm recovery rate in recovered sperm pellets can be maximized with no apparent detrimental effects on semen quality.[11]

For some stallions, sperm quality is intrinsically poor, owing to testicular/epididymal dysfunction or toxic seminal plasma. In such circumstances, semen quality can be decisively enhanced by centrifugation following passage through a density-gradient solution.[12-13] When density-gradient centrifugation is performed, sperm harvest can be reduced substantially. As such, it can be necessary to conduct a low-dose insemination technique to achieve commercially acceptable pregnancy rates when using a reduced sperm number for insemination. The following three sections address cushioned centrifugation, density-gradient centrifugation, and low-dose insemination, respectively. These approaches can be applied to clinical situations, as presented in the section on clinical cases.

Cushioned centrifugation of semen

Centrifugation of semen is oftentimes applied in equine breeding programs, so the value of improving centrifugation procedures to maximize sperm harvest, without associated sperm injury, becomes apparent. Development of a more efficient centrifugation method, i.e., one that increases post-centrifugation recovery rate while maintaining semen quality, would optimize the number of sperm in an ejaculate that are available for breeding purposes. One of the main concerns, when attempting to maximize sperm recovery through centrifugation is the adverse effect that centrifugation can have on the integrity of sperm. Typically, an increase in centrifugation time or gravitational (g) force results in an increased sperm recovery rate, but can also lead to decreased sperm motility or quality due to the mechanical forces associated with centrifugation and excessive packing of the sperm.[14] Ideally, centrifugation should result in a 100% sperm recovery rate with no resulting damage in sperm quality. Previous studies have used a variety of centrifugation forces and times in an attempt to achieve this goal; however, such protocols can lead to a 15-20% loss of sperm that could otherwise be used for breeding purposes.[11]

Recently, a cushioned centrifugation procedure has been applied to stallion semen to maximize sperm harvest without attendant injury to sperm. A non-ionic iodinated compound, iodixanol, was first reported for density-gradient cell fractionation, and has since been used as either a density gradient or as a cushion for centrifugation of sperm.[15] Investigations regarding cushioned centrifugation of stallion semen with this product have demonstrated excellent yields of sperm that were undamaged by the centrifugation process, but an optically clear centrifugation medium was required to reduce sperm losses.[16,17]

Results from our laboratory indicate that cushioned centrifugation of stallion semen in either conical-bottom tubes containing 3.5 mL of iodixanol solution as a cushion or nipple-bottom tubes containing 30 µL of iodixanol solution as a cushion can yield a high sperm harvest, while maintaining sperm function.[11] Additionally, an optically opaque extender, as is typically used in the equine breeding industry, can be used to achieve this goal. We recommend the nipple-bottom tubes over the 50-mL conical-bottom tubes for cushioned centrifugation when the sperm number in ejaculates relatively low (i.e., less than $2\text{-}3 \times 10^9$ sperm), or when it is necessary to separate more seminal plasma from sperm following centrifugation than is possible with cushioned centrifugation in conical-bottom tubes.[11] Recently, we have found that the volume of iodixanol solution can be reduced from 3.5 mL to 1 mL in conical-bottom tubes without impairing sperm harvest or semen quality.[18]

Gradient centrifugation of semen

Both continuous and discontinuous density centrifugation gradients have been used to separate sperm populations. Continuous centrifugation gradients may be more sensitive for cell selection than discontinuous density gradients, but discontinuous gradients are useful when separating cell types with known densities, such as sperm. Application of discontinuous density centrifugation gradients for sperm selection has had broad clinical application in recent years because these gradient procedures are relatively simple to perform, and have been shown to effectively separate sperm with various morphological features in an ejaculate.[13,14,19]

Using different concentrations of colloidal silica particles to form density gradients, cells are separated by specific gravity into different layers, based on isopycnic point. Density gradients are advantageous over other methods of sperm separation because the media does not penetrate cell membranes. Further, colloidal silica does not osmotically stress sperm when added to culture medium; it can be formulated to create a high specific-gravity to separate dense cells; and it has a low viscosity so as to not impede sperm cell sedimentation. The most well-known discontinuous density gradient, commercialized as Percoll™ (GE Healthcare, Little Chalfont, UK), contains colloidal silica particles coated with polyvinylpyrrolidone (PVP). Polyvinylpyrrolidone coating is used to protect cells from the potentially toxic actions of colloidal silica. Percoll™ was the most widely used discontinuous density gradient in clinical reproductive medicine until the mid-1990's when reports of endotoxin contamination led to removal of this product for clinical use. As a result, alternative discontinuous gradient solutions such as polysucrose and iodixanol, in combination (IxaPrep™, Medicult Media, Jyllinge, Denmark), and colloidal silanized silica particles (PureSperm®, Nidacon International AB, Mölndal, Sweden and ISolate®, Irvine Scientific, Santa Ana, CA) have been investigated for clinical application in assisted reproductive techniques. Colloidal solutions with silanized silica particles have been used clinically as a human assisted reproductive technology with success comparable to Percoll.

Centrifugation of equine semen through a silanated silica-particle solution has shown promise for "selecting" sperm with good motility, morphology, and chromatin quality, and enhancing the fertility of selected subfertile stallions.[19,20] Our laboratory has found that sperm recovery rate is higher, when using 15-mL capacity conical-bottom tubes, as compared to 50-mL capacity conical-bottom tubes; that use of a one-layer (EquiPure™ Bottom Layer; Nidacon International AB) gradient yielded a higher sperm recovery rate than a two-layer gradient and that gradient volumes of 2, 3 or 4 mL in 15-mL centrifugation tubes yielded similar semen quality and sperm recovery.[21]

Centrifugation of semen through a silica-particle solution, such as EquiPure™, is not a logical approach for stallions with normal semen quality, as a relatively high percentage of the sperm population can be lost to use following centrifugation. As this technique results in sperm separation based on sperm buoyancy or "isopynic point," its use is most justified when an ejaculate contains a high percentage of sperm with morphologic defects, specifically sperm with abnormal heads, abnormal midpieces, bent midpieces, bent tails, coiled tails or premature (round) germ cells. However, we have found that the technique will also improve chromatin quality in the recovered sperm population, regardless of sperm morphologic profile.[19,21] The technique can also be used when more complete separation of seminal plasma from sperm is desired, as in instances where seminal plasma appears to imparting detrimental effects on semen quality.

Low-dose insemination

The threshold number of sperm that can be used to inseminate mares and yield a commercially acceptable pregnancy rate is not known, but this number is certainly impacted by the fertility of a given stallion, and by the semen processing method that is applied (e.g., cooled storage or crypreservation) prior to insemination. Using customary methods, mares are inseminated with 200-500x10^6 progressively motile sperm deposited directly into the lumen of the uterine body when fresh semen is used.[22,23] However, one recent study revealed no difference between insemination doses of 50 or 300x10^6 progressively motile fresh sperm.[24]

Our laboratory demonstrated that only 0.0007% of sperm that are deposited into mare uteri actually gain access into the oviducts and are available for fertilization of an oocyte. However, insemination in the tip of the uterine horn ipsilateral to an ovary containing a dominant follicle resulted in a greater percentage of oviductal sperm (77%) in the ipsilateral oviduct, as compared to uterine body insemination (54%).[25] These data indicate that more sperm gain access into the oviduct of fertilization when the insemination location is the tip of the uterine horn, as opposed to the uterine body. Others have demonstrated that excellent pregnancy rates could be achieved by videoendoscope-assisted placement of as few as $1x10^6$ sperm on the oviduct papilla of preovulatory mares when the semen was first centrifuged though a discontinuous Percoll density gradient.[26]

This breeding technique, termed deep-horn low-dose insemination, has been examined in the research setting, and applied clinically in recent years. Two techniques are most commonly used for deposition of an insemination dose on or near the oviductal papilla: 1) use of a videoendoscope (termed hysteroscope) to visually locate the papilla and permit accurate placement of semen via a long catheter passed through the biopsy channel of the insertion tube, and 2) use of a flexible catheter (usually double-lumen) in which the catheter tip is guided to a position adjacent to the papilla by manipulation per rectum prior to deposition of semen. The optimal method for low-dose insemination of mares is subject to debate. One investigator stated that hysteroscopic insemination may be justified over transrectally-guided insemination unless a stallion's fertility was excellent or sperm number exceeded $25\text{-}50x10^6$ in the insemination dose.[27] Others indicate that hysteroscopic insemination may yield better results than transrectally-guided insemination if the insemination dose contains less than $5x10^6$ progressively motile sperm.[22] Recent findings in our laboratory revealed no significant difference between the two methods when mares were inseminated with as little as $0.5\text{-}1x10^6$ sperm from a stallion with known good fertility.[28] Certainly, the hysteroscopic technique has the disadvantages of increased equipment costs, labor force, and procedure time. As such, the transrectally-guided approach would seem to lend itself more favorably to widespread application in the equine breeding industry. Heightened reproductive skill of the attending veterinarian, however, is necessary for this latter technique to be successful.

The value of low-dose insemination for improving subfertility in stallions has been questioned, although successful results with this breeding strategy exist.[19,29] The aforementioned reports involved either hysteroscopic or transrectally guided approaches for low-dose insemination.

Clinical cases

This section is provided to acquaint the reader with specific clinical cases for which the techniques of cushioned centrifugation, gradient centrifugation, and/or low-dose insemination strategies have been applied in an effort to improve reproductive performance in stallions.

Case 1

A 12-year-old Quarter Horse was admitted to the Texas Veterinary Medical Center for assessment of fertility and to determine if altered strategies of breeding management could improve pregnancy rates. According to the owner, the stallion had been fertile in previous years, but per-cycle pregnancy rate dropped to approximately 35% in the previous season when covering approximately 90 mares. Combined testicular volume of this stallion was calculated to be 184 mL. Predicted daily sperm output (DSO), as determined from testicular volume, was $3.7x10^9$ sperm, but actual DSO was approximately $1.7x10^9$ sperm; therefore, spermatogenic efficiency was estimated to be less than 50%.[30] Ejaculates contained an average of 33% morphologically normal sperm and 38% progressively motile sperm. The most common morphologic defects were bent tails (average of 35%) and abnormally shaped midpieces (average of 33%). The average percentage of progressively motile sperm following 24 hours of cooled storage of extended semen was 29%.

Centrifugation of extended semen was proposed as a means to increase sperm concentration in inseminates because sperm concentration in ejaculates was generally less than $100x10^6$ per mL (range of 28 to $109x10^6$/ml; median of $68x10^6$/ml). Extended semen from the stallion was subjected to cushioned centrifugation for 20 min. at 400 x *g*, using glass nipple-bottom centrifuge tubes to reduce seminal plasma

concentration and increase sperm concentration in resuspended sperm pellets. Reproductively normal mares were inseminated with processed semen to evaluate the effects of sperm number, storage time, and insemination method on fertility. Owing to the costs associated with a breeding trial, the client was amenable only to insemination of a small group of mares to determine if simple concentration of sperm and transrectally-guided or hysteroscopic low-dose insemination techniques would result in pregnancy rates which would allow this stallion to be commercially viable (Table 1).

Table 1. Effects of total sperm number, insemination volume, and method of insemination on pregnancy rate in mares, with semen from a single stallion.

Total sperm in inseminate ($x10^6$)	Storage time for processed semen (h)	Volume of inseminate (ml)	Method of inseminatio	Per-cycle pregnancy rate (%)
500	0	1	Transrectally-guided	4/6 (66%)
500	24	1	Transrectally-guided	4/6 (66%)
250	0	0.5	Transrectally guided	5/6 (83%)
250	24	0.5	Transrectally-guided	4/6 (66%)
250	0	0.2	Hysteroscopic	1/2 (50%)

While the number of mares inseminated in each of the five treatment groups was small, the outcome was sufficient to provide generalizations regarding treatment strategies for the subject stallion. The results suggest that no difference in fertility existed between inseminates of $250x10^6$ or $500x10^6$ total sperm. Cooled storage of semen had no apparent deleterious effect on fertility, and transrectally-guided insemination of semen yielded results that were similar to, or exceeded, that of hysteroscopic insemination. A follow-up trial with cool-transported semen and transrectally-guided low-dose insemination resulted in recovery and transfer of 5 embryos from 4 mares inseminated.

Semen from this stallion had not previously been subjected to cooled storage and transport. The stallion was discharged with recommendations to use cushioned centrifugation of semen and transrectally-guided insemination with 250 million sperm per mare initially, with the prospect that sperm number in inseminates could be lowered if initial pregnancy results were favorable. In addition, the owner was informed of the potential value of cool-stored semen. A decision was made to breed mares the following season primarily with fresh processed semen. A total of 132 mares were bred over 170 cycles, with cool-transported semen used on only 3 occasions. The stallion achieved a seasonal pregnancy rate of 85% with an average of 1.5 cycles per pregnancy (i.e., a per-cycle pregnancy rate of 66%).

Case 2

A 6-year-old Quarter Horse stallion was admitted to the Texas Veterinary Medical Center for a breeding soundness examination, and for potential therapeutic approaches to improve his fertility. The stallion was administered an orally active progestogen, altrenogest (Regu-Mate®; Intervet/Schering-Plough Animal Health, Millsboro, DE), on a daily basis during his four-year athletic career. The dosage of altrenogest administered was unknown to the owner of the stallion. Only one mare was pregnant from the first eight mares inseminated with entire ejaculates, resulting in a 12% per-cycle pregnancy rate.

The stallion had a total testicular volume of 134 mL and a predicted DSO, based on testicular volume, of $2.5x10^9$ sperm. Actual DSO for the stallion was approximately $1x10^9$ sperm, so spermatogenic efficiency was determined to less than 50%. Ejaculates contained an average of 15% morphologically normal sperm and 34% progressively motile sperm. The most common sperm morphologic defects were abnormally shaped midpieces (average of 28%) and bent tails (average of 22%).

After consultation with the owner, a decision was made to inseminate a small group of reproductively normal mares by low-dose insemination techniques. Semen-processing techniques consisted of cushioned centrifugation only or cushioned centrifugation, followed by density-gradient centrifugation through a silanated silica-particle solution (EquiPure™, Nidacon International AB; Top Layer and Bottom Layer; i.e., two-layer approach) for 30 min. at 200 x *g*. The density-gradient centrifugation procedure was used in an attempt to improve semen quality through separation of spermatozoal morphologic types based on isopycnic point (i.e., buoyancy). Results of the clinical trial are provided in Table 2.

Pregnancy rates appeared to improve as insemination dose by transrectally-guided technique increased from 15 to $100x10^6$ progressively motile sperm, suggesting a dose-dependent effect of insemination number on fertility. Following cushioned centrifugation, no distinct advantage was gained by using hysteroscopic insemination, as compared to transrectally-guided insemination in the tip of the uterine horn. Insemination in the tip of the uterine horn, however, yielded a higher pregnancy rate than standard insemination in the uterine body when the inseminate contained $100x10^6$ progressively motile sperm. Further processing of semen through EquiPure™ also appeared to improve pregnancy rates, in comparison with only cushioned centrifugation, when the inseminate contained $50x10^6$ progressively motile sperm.

Table 2. Effects of semen-processing technique, method of insemination, and number of progressively motile sperm in inseminates on pregnancy rate in mares, with semen from a single stallion.

Centrifugation technique	Method of insemination	Progressively motile sperm in inseminate ($x\ 10^6$)	Inseminate Volume (mL)	Per-cycle pregnancy rate (%)
Cushioned method	Transrectally-guided	15	1	0/6 (0%)
Cushioned method	Transrectally-guided	50	1	2/6 (33%)
Cushioned method	Transrectally-guided	100	1	5/6 (83%)
Cushioned method	Hysteroscopic	50	0.1	3/8 (38%)
Cushioned method	Standard uterine body	100	1	2/6 (33%)
EquiPure™(two-layer)	Hysteroscopic	50	0.1	6/8 (75%)

The stallion was discharged from the hospital with instructions to breed mares with a low-dose insemination technique, using a minimum of $100x10^6$ progressively motile sperm following cushioned centrifugation or $50x10^6$ progressively motile sperm following centrifugation in EquiPure™. We also proposed that the insemination dose with EquiPure™-treated semen might be lowered, without impacting fertility negatively, but that this possibility should be tested before it could be recommended for commercial purposes. We did not test the fertility of cool-stored semen so could not offer recommendations regarding its use. The owner indicated that the stallion had normal fertility the following breeding season when mares were inseminated by a transrectally-guided technique using semen previously subjected to cushioned centrifugation. The precise mare book, insemination doses, and fertility statistics were not available for inclusion in this communication.

Case 3

A 4-year-old Quarter Horse stallion was admitted to the Texas Veterinary Medical Center for evaluation of breeding soundness following his first season at stud where he achieved a 59% seasonal pregnancy rate when covering 165 mares. Approximately one-half of the mares were bred with cool-transported semen. During that breeding season, progressive sperm motility was estimated to be 70% for 76 of the 84 semen collections performed, and the average total sperm number in the 84 ejaculates was approximately $5.349x10^9$ sperm. The sperm concentration was estimated to be less than $100x10^6$ sperm/mL for 67 of the 84 ejaculates collected.

The testicular volume of this stallion was determined to be 225 mL, resulting in a predicted daily sperm output of $4.6x10^9$ sperm, assuming the testes were producing sperm with normal efficiency. Four ejaculates were collected from the stallion. The stallion's actual sperm output on the fourth daily collection was $3.67x10^9$ sperm, so spermatogenic efficiency was considered to be below normal. The percentage of morphologically normal sperm in ejaculates averaged 38%, and the percentage of progressively motile sperm averaged 27%. The most common morphologic defects were abnormally shaped midpieces (average of 30%) and bent tails (19%).

Semen from three ejaculates was processed by cushioned centrifugation, or by cushioned centrifugation followed by centrifugation in EquiPure™ Bottom Layer. Semen was evaluated for sperm motion characteristics immediately following each processing step, and following 24 hours of cooled storage. The effect of seminal plasma was also evaluated. The sperm morphologic profiles of unprocessed (raw) semen and EquiPure™-processed semen were also compared. Data from a representative ejaculate are provided in Tables 3 and 4.

From these data, one can surmise that the EquiPure™ centrifugation procedure considerably improved the semen quality of this stallion, as measures of sperm-motion characteristics and sperm morphology were enhanced with this procedure. The increase in the percentage of morphologically normal sperm following EquiPure™ Bottom Layer centrifugation was primarily attributable to reduced percentages of abnormal (irregular or bent) midpieces, and bent tails. For this ejaculate, and others evaluated from this stallion, sperm velocity was increased when seminal plasma from the test stallion was replaced with that obtained a fertile control stallion. For all treatments in Table 3, sperm motility values did not change appreciably following 24 hours of cooled storage, as compared to that evaluated prior to storage.

A follow-up fertility trial was conducted with semen from this stallion to determine if insemination of mares with EquiPure™-processed semen would yield commercially acceptable pregnancy rates. Ten reproductively normal mares were inseminated in this trial; each of five mares was inseminated once with $100x10^6$ total sperm, and each of five mares was inseminated once with $200x10^6$ total sperm. The test stallion's seminal plasma was replaced with that of a fertile donor stallion. Seminal plasma from the donor stallion was procured by centrifuging raw semen for 1000 x *g* for 15 minutes, followed by filtration of supernate through tandom 5.0- and 1.2-micron pore-size nylon filters to remove any remaining sperm from the seminal plasma. One-ml aliquots of seminal plasma were frozen in vials at -80°C prior to use. Inseminate volumes ranged from 0.25 to 0.58 mL, and a transrectally-guided low-dose insemination technique was used. The per-cycle pregnancy rates were 100% (5/5) for mares inseminated with $100x10^6$ total sperm, and 4/5 (80%) for mares inseminated with $200x10^6$ total sperm. Two mares, one in each treatment group, experienced double ovulations, and each of these mares was diagnosed with twin pregnancies. As such, pregnancy rate per ovulation was 100% (6/6) for mares inseminated with 100×10^6 total sperm, and 5/6 (83%) for mares inseminated with $200x10^6$ total sperm. Based on the post-centrifugation recovery rate of sperm in this trial, the stallion would have had sufficient semen to breed 17 mares per ejaculate if mares were to be inseminated with $100x10^6$ total sperm.

Table 3. Effects of semen processing protocol (simple dilution, cushioned centrifugation, or EquiPure™ Bottom Layer centrifugation), source of seminal plasma (same stallion or fertile control stallion), and storage time (0 or 24 h) on measures of sperm motility.

Centrifugation Technique	Source of seminal plasma	Sperm concentration (x106/ml)	Storage Time (h)	Total Motility (%)*	Progressive Motility (%)*	Curvilinear Velocity (μm/s)†
Simple dilution	Same	30	0	54	21	176
Cushioned centrifugation	Same	30	0	59	33	121
EquiPure™	Same	30	0	90	77	146
EquiPure™	Control	30	0	93	82	224
Simple dilution	Same	30	24	50	20	136
Cushioned centrifugation	Same	200	24	59	29	152
Cushioned centrifugation	Same	30	24	56	33	135
EquiPure™	Same	200	24	87	72	141
EquiPure™	Control	200	24	89	73	208

†Measurements for total motility (%), progressive motility (%), and curvilinear velocity (μm/s) were performed by computer-assisted sperm motion analysis.

Table 4. Effect of semen centrifugation through EquiPure™ Bottom Layer on sperm morphologic features, as viewed by differential-interference microscopy at 1250 x magnification.

Sperm morphologic feature (%)	Unprocessed (raw) semen	EquiPure™-processed semen
Normal	40	76
Abnormal heads	5	1
Abnormal acrosomes	1	1
Tailless heads	3	2
Proximal protoplasmic droplets	10	5
Distal protoplasmic droplets	13	5
Abnormal (irregular) midpieces	28	6
Bent midpieces	13	3
Bent tails	19	5
Coiled tails	1	0
Premature (round) germ cells	1	0

For the following commercial breeding season, 212 mares were inseminated, primarily using fresh EquiPure-treated semen with seminal plasma from a fertile donor stallion. The stallion achieved a seasonal pregnancy rate of 91% in an average of 1.61 cycles per pregnancy (i.e., a per-cycle pregnancy rate of 62%).

Summary

Equine veterinarians may encounter owners of stallions who are seeking methods that can improve stallion breeding performance. Breeding and semen-manipulation strategies can be applied to maximize the fertility of these stallions and to extend their productive life. Cushioned centrifugation, density-gradient centrifugation, and low-dose insemination techniques can be used effectively in some

instances for reproductive management of these stallions. Such semen-processing and breeding strategies are best evaluated outside the commercial breeding season by conducting clinical fertility trials prior to applying these in a commercial setting. Recipient-mare herds retained by embryo-transfer facilities are an excellent source for such trials. The information gained provides useful information for the stallion owner/agent as they prepare for a forthcoming breeding season.

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The stallion breeding soundness evaluation: revisited

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Abstract

The stallion breeding soundness evaluation (BSE) was originally formalized in 1983 in pamphlet form by the Society for Theriogenology and called the Manual for Clinical Fertility Evaluation of the Stallion. Since that time it has provided an invaluable guideline for practitioners to critically evaluate the reproductive capability of a stallion by providing an outline on how to perform and interpret those findings. The horse breeding industry has changed and new information has become available concerning aspects that affect the performance and interpretation of the BSE. This proceeding discusses several aspects of the BSE including the evaluation of sperm quality, interpretation of BSE findings and prediction of fertility.

Keywords: Stallion, sperm, motility, morphology, breeding soundness, fertility

Introduction

The guidelines for performing a stallion breeding soundness evaluation (BSE) were introduced in pamphlet form in 1983 by the Society for Theriogenology.[1] In performing a thorough evaluation of the stallion the practitioner is able "to eliminate from consideration…or at least alert the owner of potential problems". It is further stated "the examination will assist identifying the cause(s) of reduced fertility and the findings used to develop guidelines for management of the stallion to enable it to achieve its maximum fertility".

The intent of the stallion BSE is different from those performed on food animals in which the goal is to identify those males that fall below a specific threshold for sperm quality and other factors and eliminate them from the population. In contrast, the goal of the stallion BSE is to identify the cause and pursue, in many cases, a resolution of the problem. This approach is often questioned with the concern that the cause of the reduced fertility/subfertility may be hereditary in nature and therefore the trait should not be propagated by breeding the stallion. While the level of reduced/subfertility in the stallion population due to hereditary causes is unknown, non-hereditary causes such as illness and advanced age are well established and likely account for the majority of inherent stallion subfertility. In addition, other causes of "stallion subfertility" often overlooked include mare and management limitations.

The Manual suggested that the results of the BSE would classify a stallion as Satisfactory, Questionable, or Unsatisfactory based on the ability of a stallion to "render pregnant" 75% of 40 mares by natural cover, or 120 mares by artificial insemination. This suggests a 50%/cycle pregnancy rate if each mare is exposed to the stallion for at least two estrous cycles. These book sizes of 40 and 120 mares were not randomly chosen but rather resulted from what at the time, were the number of shares sold at the time stallions were syndicated (40 for Thoroughbreds; 120 for Standardbreds). Therefore, these recommendations were intended to accommodate industry expectations, primarily Thoroughbred and Standardbred, and provide conservative endpoints to evaluate and render an opinion the reproductive capability of a stallion. They were not, however, based on a biological threshold for fertility, nor are the sperm quality guidelines in the manual directly related to a specific endpoint (i.e. 75% pregnancy rate in a book of 40 mares).

The BSE Manual has provided the veterinary clinician an invaluable framework to examine, diagnose, and treat reproductive problems in the stallion. Since 1983 the horse breeding industry has been dynamic, with the introduction and common application of cooled-shipped and frozen semen. In addition, the Thoroughbred industry has increased the number of mares (i.e. 100-200 mares) to which the more popular stallions are bred. These changes have created challenges that were not addressed in the BSE manual such as how to determine the number of mares a stallion can be bred to for those larger book sizes. The manual recommendations for book size, particularly for Thoroughbred stallions (40 mares),

was probably a very modest challenge to the fertility of even stallions of lesser fertility as the authors of the Manual were probably aware. Another area of interest includes the evaluation of sperm quality and how it relates to fertility. At the time the manual was published, evaluation of sperm quality primarily involved fresh semen, since then however, the expanded use of cooled-shipped and frozen semen has raised questions about the evaluation of these sperm types. The number of sperm quality assays and the ability of these assays to describe sperm quality objectively have advanced considerably as well as the ability of these assays to describe the relationship of sperm quality to fertility.

Based on the expanded knowledge, it would benefit the discipline to update concepts and guidelines set forth in the BSE manual. This proceeding is not intended to be all-inclusive but rather to provide critique and suggestion regarding several aspects of the stallion BSE.

The case against progressive motility

It has been suggested that progressive sperm motility is "essential" for fertility in the stallion[2] as well as the "most critical aspect of motility"[1], however, "progressive" motility has not been shown to be more important than *total* motility or other measure of sperm quality.

Sperm motility is historically the most common assay performed to determine sperm quality, particularly when samples are evaluated as part of routine semen collection and processing, commonly in concert with breeding activities or the BSE. Sperm activity can be described as either *total* or *progressive*, but for diagnostic purposes the concept of *progressive* motility was initially introduced and has been adopted.[3,4] Historically, sperm motility has been determined using the "subjective method" by visual microscopic examination. While it is unclear why the concept of "progressive motility" was adopted, it appears to convey an additional subjective quality measure that indicates sperm that are "straight" and "fast enough", which further suggests a "goodness" that "non-progressive" sperm do not possess. This has translated into a more "precise/sensitive/predictive" index of fertility. While there have been many studies that use progressive sperm motility as their endpoint to measure sperm quality, there have been none that have validated this measure to determine if it "means" anything more than simply *total* motility or other assays of sperm quality. Sperm motility was initially adopted as a measure of sperm quality because microscopy was the only method available to evaluate sperm, however, there have been additional assays developed that can add to the clinicians ability to render a diagnosis. Sperm motility has been adopted "because we can" rather than because many assays have been compared and it was the single assay that rose to the top as the "best".

The definition of total motility is straightforward and is simply the percentage of sperm that display any type of motility regardless of the "quality" of that motility. In contrast, *progressive* motility, while conceptually well-defined, is practically without definition. Conceptually, *progressive* means "moving *relatively* straight across the microscopic field" or "*actively* moving forward",[1] however, practically, these vague definitions have resulted in an assay that lacks inter-operator repeatability resulting in confusion regarding the "quality" of a semen sample. While it may be considered an academic discussion the inability to describe the threshold that separates a progressive from a non-progressive sperm is a clinically critical question, because that separation is what has been used to infer the difference between a fertile and infertile sperm. Fertile sperm are straight and fast (progressive), while infertile sperm are not. More recently computerized systems have been introduced to provide more "objective" results. One limitation of this assay is repeatability, or the ability of the assay (progressive motility) to give the same measure when different individuals measure the same sample. A previous study determined that the coefficient of variation was high for subjective assessment of progressive sperm motility (20%) compared to total sperm motility (10%) and determined that five ejaculates were required for an error rate of 10% for progressive motility while only one sample was needed for total motility.[5] In another study,[6] the between-breeding season coefficient of correlation was higher for total than progressive sperm motility for both subjective ($r = 0.63$ vs. 0.48) and computer assisted motility analysis ($r = 0.63$ vs. 0.34). In addition, both total and progressive motility were able to discriminate high and low fertility stallions while mean spermatozoa linearity (progressive movement) was not.

The lack of repeatability of progressive motility is easily demonstrated if a sperm sample is projected on an overhead screen to a group of clinicians or students and they blindly record *total* and *progressive* motility values. There will often be considerable agreement between total motility scores but far less for progressive motility.

Why is a discussion about progressive motility anything more than an academic exercise? It has been widely adopted in both lay and veterinary jargon as the sole determinant of a stallion's reproductive capability. Instead of thoroughly evaluating a semen sample, using assays that are more repeatable and diagnostic, both the clinicians and researchers continue to refer to this poorly defined assay to describe sperm quality. In addition, a stallion's present or future fertility "reputation" is often based on the results of this assay.

The advent of computerized motility analyzers would seem to have solved the problem of assay repeatability, since "progressive" can be objectively defined by machine settings. Unfortunately, having a repeatable assay is of little use if the assay is not interpretable or does not relate to a relevant biological endpoint such as fertility.

A threshold for "adequate" progressive motility has been randomly set for frozen-thawed stallion semen at 30% and sometimes (European Union) at 35%. This threshold too is arbitrary and is often the sole criterion determining whether a stallion's ejaculate "passes" or "fails" the post-thaw motility assay. Compounding this problem is that other more relevant sperm quality measures (morphology, longevity of motility, viability) are ignored. And perhaps the single most important measure, the *total* number of sperm inseminated, is not controlled at all.

Why is this relevant to the clinician? A common clinical question is "what do these sperm assays mean?" Many clinicians are familiar with a stallion that had low sperm motility in his ejaculate that is "fertile" or the stallion that has excellent sperm quality yet whose fertility is "low". Often the conclusion is that sperm quality assays means little because of the lack of correlation. This conclusion is often drawn with little knowledge of the breeding circumstances (mare and management) and the assumption is then made that these "other" factors have no influence on the reported fertility outcome.

As diagnosticians "low" progressive motility is used to diagnose a stallion with a history of inadequate fertility. In effect we are saying this stallion has *pathologically* abnormal sperm quality sufficient to explain the fertility reduction described in the history. The lack of "progressive" sperm motility, however, is commonly not pathologic. Non-progressive sperm motility can be a transient occurrence caused by temperature or subtle osmotic changes. In addition, there are stallions whose sperm just circle, but who are highly fertile, perhaps due to the abaxial position of the midpiece attachment on the sperm head. A closer look at the sperm quality of these stallions will often find that they have a high percentage of morphologically normal sperm, high viability and DNA quality, and that their longevity of total sperm motility does not change after cooled storage, characteristics that are consistent with a fertile stallion. Longevity of sperm motility in the cooled state is a useful assay to determine whether a sperm sample with "low progressive motility" is pathologic. If the total motility is maintained for 24 hours, it is less likely that the "low" progressive motility is a fertility limiting factor.

Suggestion:

Sperm motility is recognized as an important factor that plays a role in fertility, but it should not be used alone as a determinant of a stallion's reproductive potential simply because of its historic application. The consequences of incorrectly diagnosing a subfertile stallion simply because of low progressive motility particularly in the face of high total motility, are profound and when incorrect result in a loss of credibility to the profession and the procedures that are so critical to our discipline. We currently have many other assays available to supplement the evaluation of sperm motility as a measure of sperm quality to provide a more thorough evaluation of sperm quality and render a clearer diagnosis.

Morphologic evaluation of sperm quality

Enumerating the shape of a stallion sperm population is an important part of the BSE. The percent morphologically normal is used to calculate the total number sperm that are progressively motile

and morphologically normal. This number, in the second ejaculate (at least 1.0 billion in the month of December), is considered an important value when determining the final classification status (Satisfactory, Questionable, Unsatisfactory). A relevant criticism of this composite number is that both progressively motile and morphologically normal sperm are, to a certain degree, measuring the same endpoint (i.e. a good sperm) and therefore the total number of sperm that a stallion has in the second ejaculate is being evaluated twice and that stallion may unfairly be "downgraded" in classification status. It should be recognized that there are sperm abnormalities such as proximal droplets, distal droplets, as well as abnormal heads and midpieces that may be "progressively motile" and if not accounted for in the composite number may artificially inflate a stallions "normal" sperm number. While this is an imperfect number, an alternative would be to replace progressively motile sperm with total motile sperm.

Evaluation of sperm morphology in the stallion is performed differently than other species that originally adopted the primary, secondary, and tertiary nomenclature to describe the origin of the abnormality (testis, epididymis, iatrogenic). In contrast, the stallion BSE identifies the specific sperm shape (abnormal head, detached head, coiled tail, etc). While the Manual stated that "the numbers of morphologically abnormal or non-motile forms is relatively unimportant concerning stallion fertility and are important only in calculating the number of normal sperm" the type of abnormality may be important diagnostically, particularly when the percent of normal sperm is low.[7] As an example, a stallion may have a low percent normal sperm due to transient defects such as distal droplets (a better prognosis) or due to defects associated with sperm production in the testes such as abnormal heads and midpieces (potentially a worse prognosis). Diagnostically, identification of specific abnormalities not only suggest the origin of the defect, but may narrow the diagnosis and aid the clinician in developing a prognosis and treatment.

The evaluation of sperm morphology may be the single most important sperm assay available to the practitioner because sperm shape in most cases is not altered by iatrogenic causes such as improper handling, and the results reflect what the stallion is ejaculating, without the bias inherent in sperm motility evaluations. It also lends itself to remote evaluation since sperm samples can be fixed on-farm and transported to specialists for evaluation and interpretation.

Fertility and prediction

One challenge of the BSE is that the practitioner/owner would like a prediction of fertility. The best measure of fertility, as is often stated, is breeding females and determining pregnancy. This is however, simplistic because the level of "satisfactory" fertility is difficult to define in the horse industry due to the varying owner expectations. Some owners/syndicates may view acceptable fertility based on the number of foals produced to satisfy an economic endpoint, while others may be view acceptable as the production of an individual foal to continue a particular line from an old subfertile stallion or mare, to satisfy an individual goal.

The goal of most examinations is the prediction of an outcome. In the case of the stallion BSE, fertility prediction based on a set of measureable variables would be desirable. It is commonly stated that the BSE, regardless of species, cannot predict fertility, but rather is better at identifying the low/sub-fertile individual. This conclusion is often based on the reliability of the clinical outcome.

For instance, stallions with poor sperm quality are more likely to also have low fertility because regardless of the effects of mare or management, sperm quality will be the factor that limits fertility. Therefore, there is essentially only one outcome for a male with poor sperm quality and that is a reduction in fertility making the clinical diagnosis "correct" most of the time. In contrast, the stallion with "good" sperm quality has two options. One, fertility is representative of the level of sperm quality (i.e. good, excellent); or two, fertility is not representative and thus sperm quality is perceived as not "predictive" of fertility. The latter example is what leads to the conclusion that the BSE cannot predict fertility, which is unfortunate because in this case measuring the role of the female and management is critical, but often ignored, leading to the conclusion that the sperm test is no good. It is therefore likely that if the fertility of mare quality and management could be measured and accounted for, that good sperm quality *would be* predictive of fertility. A recent study highlighted the prominent role that mare (increasing mare age) and

a management factor (the use of cooled-shipped semen) had in reducing fertility in a group of stallions in which sperm quality was uniformly high and did not affect fertility.[8]

Previous authors have described the relationship between fertility groups (high and low) and sperm motility[6] or morphology[9] as well as the relationship between morphologic features and fertility.[7] A recent study[10] compared the relationship between sperm motility, morphology and three fertility groups (high, average, and below average; Table). This study described the relationship between three fertility measures (seasonal pregnancy rate, percent pregnant per cycle and pregnant per first cycle) and sperm quality measures. The only sperm quality measure able to discriminate groups of high and low seasonal pregnancy rate was percent total motility. However, when percent pregnant per cycle and percent pregnant per first cycle were used, numerous sperm quality measures discriminated different fertility groups. Why then are there stallions with excellent sperm quality that do not have good fertility? These stallions truly exist, such as those whose acrosomes do not react in the face of excellent sperm quality (motility, morphology).[11]

An additional factor that limits our ability to predict fertility is the fertility measure itself. Seasonal pregnancy rate is a common measure used to describe fertility compared to pregnant per cycle or pregnant per first cycle. Yet when these three measures were used to describe fertility in a group of clinically fertile stallions that were divided into high, average and low fertility, all three of the seasonal pregnancy rate (SPR) groups had a stallion with a 100% seasonal pregnancy rate (Table 1).[10] Clinically, this suggests why some stallions are "fertile" based on SPR while their sperm quality suggests they are less fertile based on a more sensitive measure such as percent pregnant per cycle or pregnant per first cycle. The limitation of seasonal pregnancy rate as a measure of fertility is that it is an accumulation of breedings and not a measure of breeding efficiency allowing less fertile stallions to "catch up" by breeding mares more cycles.

Suggestion

Poor sperm quality, by itself, can be the limiting factor in fertility, but good sperm quality does not assure that other factors (mare, management) are also optimal and not in themselves a limiting factor. Instead of this finding being included as part of the diagnosis (i.e. this is actually a fertile stallion, look elsewhere for the cause of subfertility), the conclusion is that this is truly a subfertile stallion and this sperm assay is inadequate. If you change the assumption from the "the assay is inadequate" to "the assay is always inherently correct" it means the diagnostician must then pursue another reason for the "stallion's" subfertility.

There have been few studies that have compared fertility levels with sperm quality features. A common outcome is that the relationship (usually correlative) may be low, but statistically significant. The conclusion drawn from these results is that the relationship of sperm quality to fertility is of little use clinically (i.e. not predictive). This is unfortunate because included in these "low" correlations are mare and management factors that are not accounted for in the analysis. If these factors were accounted for, correlations would likely be "higher".

The dilemma of classification

Similar to the bull, a classification system (Satisfactory, Questionable, Unsatisfactory, Classification Deferred) is provided in the BSE manual. The two classification systems (bull and stallion) however, have different intents. The intent of the bull system is to identify those individuals that, based on sperm criteria as well as potential physical and behavioral characteristics, do not attain a certain threshold, and eliminate them from the breeding population. The stallion BSE, as mentioned earlier, has the intent of providing a framework to perform a thorough examination allowing the practitioner to identify causative fertility limiting factors. The classification categories are intended to provide a basis for the practitioner to give perspective (small testes, average sperm quality) and interpretation about a stallion's reproductive capability. The classification system is not a Pass/Fail system which suggests that stallions that Fail should or cannot be bred. The concept of Fail may have long-standing effects on a stallion's reputation that reduce the stallion's economic viability, particularly if the fertility limiting

condition is transient. The classification system is not intended to provide a platform for the practitioner to pass judgment as to whether a stallion should or should not be bred. This approach is particularly important regarding conditions that may be *potentially* genetically based (cryptorchidism, low sperm numbers or quality) versus those that are *definitively* genetically based (hyperkalemic periodic paralysis [HYPP], hereditary equine regional dermal asthenia [HERDA]). As information becomes available about conditions like cryptorchidism it may be apparent that they are multfactorial (genetic and environmental) rather than exclusively genetic in origin.[12] Even in the case of those conditions that have been established as genetic, the ultimate resolution should be the purview of the breed associations. It should be the responsibility of the practitioner to provide accurate information regarding the condition so the owner and breed association can render a responsible opinion.

The Society for Theriogenology Manual for Clinical Fertility Evaluation of the Stallion has been an extremely useful tool for the veterinary practitioner since it was introduced in 1983. There has been considerable information available since the introduction of the manual that can would be useful in updating and further promoting this valuable publication.

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Table. The mean (± SD) and range () for stallion sperm motility and morphology variables for the percent mares pregnant / cycle. (reprinted from Love, Theriogenology 2011)

	1	2	3
Seasonal Pregnancy rate (%)	97 ± 4 (90-100)	86 ± 10 (50-100)	61 ± 29 (12-100)
Pregnant / cycle (%)	91 ± 10 (75-100)	56 ± 6 (45-74)	32 ± 13 (8-45)
Pregnant / first cycle (%)	91 ± 10 (75-100)	58 ± 16 (0-88)	34 ± 14 (0-50)
Sperm motility values			
Total motility (%) [1]	83 ± 5 (76-91)[a]	76 ± 12 (44-92)[a]	48 ± 21 (18-66)[b]
Progressive motility (%)[2]	77 ± 6 (64-85)[a]	71 ± 12 (43-88)[a]	44 ± 20 (16-63)[b]
Path velocity (μ/s)[5]	196 ± 26 (163-230)[a]	190 ± 19 (157-241)[a]	162 ± 41 (104-194)[b]
Progressive velocity (μ/s) [6]	174 ± 20 (146-207)[a]	171 ± 17 (143-208)[a]	139 ± 34 (88-166)[b]
Morphology			
Normal[1]	67 ± 8 (50-76)[a]	48 ± 15 (17-83)[b]	41 ± 27 (7-85)[b]
Abnormal heads	9 ± 6 (1-22)	12 ± 9 (0-45)	17 ± 13 (1-43)
Detached heads[2]	2 ± 2 (1-8)[a]	2 ± 2 (1-10)[a]	6 ± 11 (0-44)[b]
Proximal droplets[3]	8 ± 3 (5-14)[a]	20 ± 14 (2-51)[b]	25 ± 18 (4-60)[b]
Distal droplets[4]	6 ± 4 (2-16)[a]	8 ± 7 (0-28)[a]	2 ± 2 (1-7)[b]
Bent midpieces	1 ± 1 (0-4)	0.2 ± 0.4 (0-1)	1 ± 5 (0-17)
General midpiece abnormality[5]	6 ± 4 (1-14)[a]	7 ± 6 (1-29)[ab]	10 ± 6 (2-26)[b]
Hairpin tail	4 ± 3 (0-9)	4 ± 4 (0-16)	5 ± 6 (0-20)
Coiled tail[6]	1 ± 1 (0-3)[a]	2 ± 2 (0-7)[a]	5 ± 5 (0-14)[b]
Premature germ cell	1 ± 2 (0-6)	1 ± 2 (0-8)	2 ± 2 (0-7)

Percent pregnant / cycle groups – Group 1- ≥ 76% and ≤ 100%; Group 2- ≥ 46% and < 76%; Group 3- ≥ 0% and < 46%.
1-P< 0.0001
2-P<0.001
3-P<0.0002
4-P<0.04
5-P<0.04
6-P<0.008

Management of partial obstruction of ejaculatory ducts in stallions: etiology, diagnosis, and treatment

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Abstract

Fertility problems in stallions have a variety of causes. Despite decades of clinical investigations, many of these causes still remain unknown. Therefore, treatment often is symptomatic rather than causative, and rarely leads to complete and permanent cure of the problem. This paper describes etiology, diagnosis and treatment of one of the little known disorders in stallions, which may have a significant impact on stallion's breeding career.

Keywords: Stallion, fertility, ejaculatory duct, obstruction, utriculus masculinus

Introduction

Partial obstruction of the ejaculatory ducts is an under-diagnosed disorder in stallions, which causes a variety of clinical signs, and affects their fertility. More frequent use of high-resolution transrectal ultrasonography (TRUS) in recent years resulted in recognition of this condition in stallions with fertility problems. There is no one pathognomic sign of partial obstruction of the ejaculatory ducts in stallions, which makes this disorder very challenging to diagnose. Furthermore, the pathogenesis of partial obstruction of the ejaculatory ducts, and the impact of this disorder on stallions' fertility are not very well understood. Therefore, this paper discusses the anatomy of the ejaculatory ductal system, as well the symptomatology, semen characteristics, and TRUS findings in stallions with a presumptive diagnosis of partial obstruction of ejaculatory ducts.

Anatomy

The vas deferens in stallions travels from the tail of the epididymis to the ejaculatory duct. After entering the abdominal cavity, it turns backward, and runs towards the pelvic cavity. It is attached to the lower part of the lateral wall of the pelvis by the urogenital fold.[1] The vas deferens is freed from the urogenital fold at the level of the neck of the bladder, where both vasa lie very close together, separated by a very narrow space, or by the remnant of the Müllerian duct, often called uterus masculinus (Figure 1).[1] Furthermore, the vasa are flanked laterally by the necks of the vesicular glands. All these structures dive underneath the isthmus of the prostate, travel to the caudal edge of this gland, and continue through the wall of the urethra. Each vas deferens joins the excretory duct of the collateral vesicular gland under approximately 50° to form ejaculatory duct, which opens on the summit of the urethra, called colliculus seminalis. In some instances (approximately 15% of stallions) the vas deferens may open separately, just next to the excretory duct of the vesicular gland.[1]

The vas deferens in stallions is a relatively thick tube with a narrow lumen with the exception of its thickened glandular part called ampulla ductus deferentis. The ampulla is formed where the vas deferens is curving backwards towards the pelvic cavity, just before the vas travels over the urinary bladder. It consists of the numerous tubulo-alveolar glands, which completely disappear once the vas passes the isthmus of the prostate and dips to the wall of the urethra.[1] The average outside diameter of the glandless part of the vas deferens in the stallion is 6 mm, while the ampulla is approximately 20 mm thick, and 15–25 cm long.[1,2] Initially, the lumen of the vas deferens is narrow between the epididymal tail and the ampulla (1.1mm–3.8 mm); significantly expands through the ampulla (9–18 mm), and becomes spiral, with multiple, ring-shaped protrusions.[2] The lumen of the most terminal part of the vas deferens in stallions is narrow again (approximately 3 mm), with the average length of this fragment of the vas being 4.6 cm.[2]

The ejaculatory ducts in stallions are short, thin-walled tubes, only 2–3 mm long, and 6–7 mm wide.[1] This anatomy varies significantly from man, in which the ejaculatory ducts are long (1–2 cm) and narrow, which makes them prone to physical obstruction by various neighboring structures.[3,4]

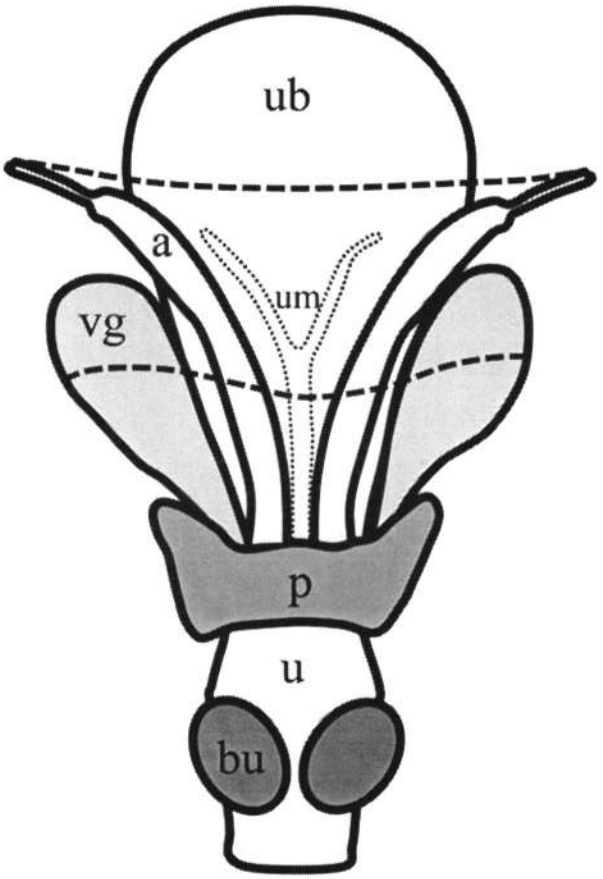

Figure 1. Diagram of stallion internal genitalia (ub–urinary bladder; a-ampulla of vas deferens; vg–vesicular gland; p–prostate; bu–bulbourethral gland; u–urethra; um-uterus masculinus)

The colliculus seminalis often contains a rudimentary structure, called the utticulus masculinus, vagina masculina, sinus prostaticus Morgagni, or alveus urogenitalis Meckel (Figure 2).[5] The utriculus masculinus in horses has a more complex origin than the uterus masculinus. While its cranial portion is a remnant of the Müllerian duct, the most caudal portion has histological features similar to the vagina and is derived from the urogenital sinus.[6] This structure forms a prominent diverticulum on the colliculus seminalis, which may be 5–7 cm long, and opens between the orifices of the ejaculatory ducts.[6] Occasionally it connects with the ejaculatory duct, or ends blindly.[1]

Etiologies

Ejaculatory duct obstruction can be either congenital or acquired. The only congenital cause of this disorder in stallions described to date is a utricular cyst, also called the midline cyst of the colliculus seminalis (Figure 2c).[7] Acquired causes may be secondary to the formation of the thick gel plugs in the excretory ducts of the vesicular glands, which are later pushed into the ejaculatory ducts. Furthermore, calculi can be formed in the ejaculatory ducts, the excretory ducts of the vesicular glands, and in the terminal portions of the vasa deferentia. In men, trauma, infectious, or inflammatory causes have been described.[4]

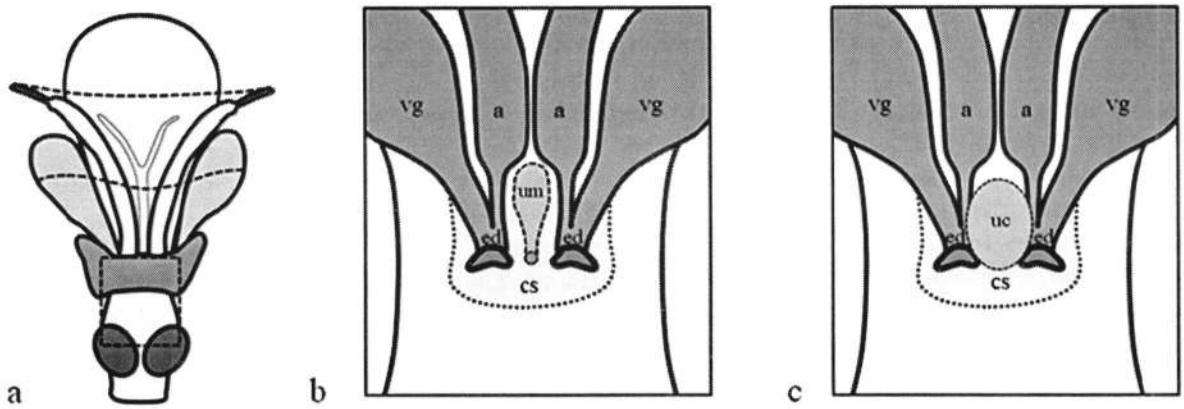

Figure 2. Diagrams of a pelvic urethra at the level of colliculus seminalis in a stallion (a–ampulla of vas deferens; vg–vesicular gland; ed–ejaculatory duct; um-utriculus masculinus; uc–utricular cyst; cs–colliculus seminalis)

a. Large view of the accessory sex glands–rectangular box enlarged in b and c
b. Magnified view of the ejaculatory apparatus with normal utriculus masculinus
c. Magnified view of the ejaculatory apparatus with utricular cyst

Utricular cysts, even though they have a congenital origin, may change in size and character of the epithelium due to the various levels of hormonal stimulation during puberty, the breeding season, or the aging process.[8] Therefore, the impact of this structure on the ejaculatory process is not consistent and may vary during the lifespan of the animal. Large utricular cysts occupy the major portion of the colliculus seminalis and directly compress the structures which are traveling through the colliculus seminalis to the ejaculatory orifices. Ejaculatory ducts are especially vulnerable since they have thin walls and can collapse easily. On the other hand, however, even severe compression rarely completely obliterates their lumen due to the fact that they are wide and do not have a distinct sphincter.

The vesicular glands produce the gel portion of the ejaculate in stallions. The consistency of this last fraction of the ejaculate varies between individual animals and may be liquid, slightly gelatinous, or very thick and tapioca-like. Furthermore, the character of the gel portion may change in each individual stallion depending on the exposure to mares, social status, and frequency of ejaculation.[9] Prolonged exposure of stallions to mares, without an opportunity to ejaculate, contributes to the accumulation of the large amounts of gel in the vesicular glands which thickens with time and forms thick plugs. These plugs can partially obliterate the ejaculatory ducts and affect the ejaculatory process.

Occasionally, small calculi or very hard accumulations of spermatozoa and/or bacteria are formed in the ejaculatory ducts, vasa deferentia, or the excretory ducts of the vesicular ducts. There may be multiple, small accumulations, which have grid-like appearance, or just one or two larger structures. The pathogenesis of the formation of these accumulations is not yet known, especially when only bacteria are present, without any inflammatory cells.

Clinical signs

There is a whole spectrum of clinical signs associated with partial occlusion of ejaculatory ducts in stallions. These include periodic ejaculatory dysfunction, decreased force of ejaculation, low ejaculatory volume and sperm numbers, poor motility of spermatozoa, or hematospermia. Usually only one or two of these signs are expressed simultaneously in the affected individual. Some stallions may have periods of time when ejaculation does not occur despite multiple efforts alternating with periods when the ejaculatory process is normal. Sexual behavior is normal up to the moment just proceeding ejaculation, however urethral pulsations or tail flagging do not occur. Due to the partial occlusion of the ejaculatory ducts only small amount of semen or seminal plasma is voided to the pelvic urethra and the ejaculatory process is not triggered. The affected stallions continue thrusting, or may start thrusting again

after a short pause. Some individuals may have one to three urethral pulses and a small amount of ejaculate is expelled. However, the force of ejaculation is significantly decreased. This phenomenon is easily observed during semen collection with an open-ended artificial vagina.

Some stallions do not have any obvious ejaculatory dysfunctions, but the quality of semen is low which leads to decreased fertility or infertility. Motility of spermatozoa is affected most frequently in stallions with the physical findings consistent with partial obstruction of ejaculatory ducts. Sperm morphology is often normal, with the exception of increased sperm with cytoplasmic droplets. Furthermore, severe oligospermia is a typical feature of partial obstruction of ejaculatory ducts in stallions. The quality of semen may fluctuate without any obvious reason.

Hemospermia was also observed in some stallions with partial obstruction of ejaculatory ducts. This can be the only sign of this disorder, or it can accompany others such as ejaculatory dysfunction or poor semen quality. The amount of blood in semen varies, and can be either very small and barely noticeable or quite significant giving the semen a bright red color.

Clinical findings

Typically, stallions with suspected partial obstruction of ejaculatory ducts appear normal on physical examination of external genitalia. Testes have normal size, consistency, and ultrasound appearance. Epididymides usually appear normal on palpation and ultrasound evaluation, however, the epididymal duct can be somewhat distended, especially if there were numerous unsuccessful attempts to collect semen. Rectal palpation of the internal genitalia is rarely revealing. However, vesicular glands may feel enlarged, especially when a large amount of thick gel is accumulated in their lumina. Furthermore, ampullae are often prominent on palpation.

Treansrectal ultrasonography findings are crucial in diagnosis of partial occlusion of ejaculatory ducts. Utricular cysts are readily visualized within the colliculus seminalis. They are usually anechoic, have oval, tear, spindle, or rectangular shapes (Figure 3).[7] Some cysts have echogenic contents, which make them more difficult to detect using ultrasonography. Distended lumina of the vesicular glands or ampullae are also often seen on the ultrasound images. The contents of these glands is usually anechoic, but may become echogenic or even hyperechoic if the occlusion is severe and prevents normal ejaculation for a prolonged period of time (Figure 4). The presence of a large amount of hyperechoic contents in the vesicular glands in the absence of utricular cysts suggests accumulation of gel. Finally, small hyperechoic structures may be found in the ejaculatory ducts, the excretory ducts of the vesicular glands, or in the vasa deferentia. Occasionally, these structures are expelled during ejaculation. They may be non-cellular or contain large numbers of bacteria, spermatozoa and epithelial cells.

Urethroscopy is always performed in stallions with hemospermia. In the absence of penile pathology, urethral rents, or seminal vesiculitis, a bleeding utricular cyst should be considered. Oozing of blood from the utricular orifice may be visible during urethroscopy.

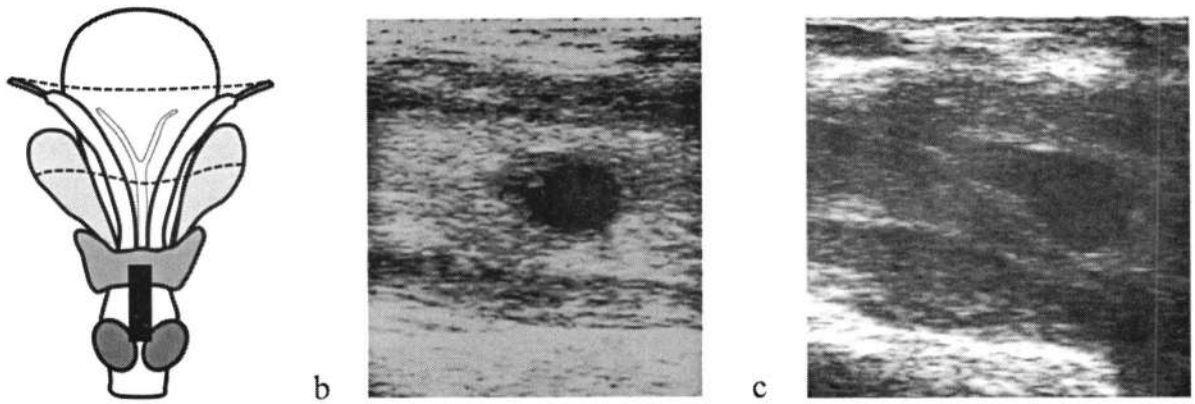

Figure 3. Ultrasound images of the utricular cyst.
a. Position of a transducer
b. Oval-shaped cyst
c. Tear-shaped cyst

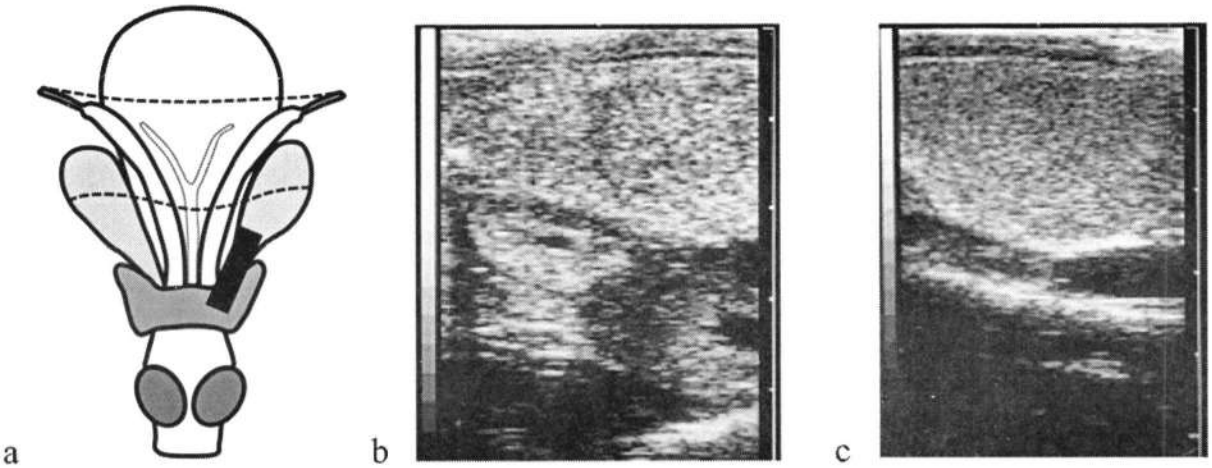

Figure 4. Ultrasound images of hyperechoic accumulation of gel in the vesicular glands in stallions.
a. Position of a transducer
b. Hyperechoic contents in the excretory duct of the vesicular gland
c. Hyperechoic contents in the fundus of the vesicular gland

Treatment

There is no specific treatment for partial occlusion of ejaculatory ducts in stallions; however, this disorder can be successfully managed. An experienced team of skilled people should work with stallions with this problem.

If the utricular cyst is compressing the ejaculatory ducts, the process of emission is significantly affected and the ejaculatory threshold cannot be reached. In order to assure that the adequate volume of fluid is voided into the urethral lumen during emission, the effected stallion should have a chance to produce enough semen and seminal plasma. Therefore, the number of natural breedings or semen collections should be limited, and teasing should be prolonged. In addition, strong stimulation during semen collection may help in reaching the ejaculatory threshold. Surprisingly, manual massage of the ampulae per rectum before ejaculation is often ineffective. However, pre-treatment with oxytocin (20 IU, iv, immediately before ejaculation) seems to enhance the chance for success. In addition, imipramine hydrochloride can be given (1200 mg, per os, two hours before ejaculation), in order to lower the

ejaculatory threshold. Interestingly, a successful ejaculation does not improve the chance for another ejaculation to occur.

The accumulations of thick gel in the vesicular glands need to be expelled in order to improve ejaculatory process in affected stallions. Manual trasrectal massage of the vesicular glands towards the colliclus seminalis, as well as treatment with oxytocin is helpful in expulsion of this gel. The volume of these accumulations may be massive. Therefore, multiple ejaculations are often necessary to completely remove the contents of the vesicular glands. The affected stallion should be placed on a regular schedule of ejaculation which will prevent future problems.

Expulsion of calculi or hard accumulations of spermatozoa and bacteria require strong stimulation and manual trasrectal massage of the ejaculatory apparatus; oxytocin treatment is often applied as well. Currently, there are no data on possible re-occurrence of this problem.

Conclusions

Partial obstruction of ejaculatory ducts in stallions occurs rarely, but may be very frustrating. It arises from several different causes, and is associated with a variety of clinical signs. To date, there is no permanent treatment for this disorder, but if properly recognized, it can be managed successfully.

A similar disorder is well known in human patients. A variety of treatments which result in a permanent cure are available.[4,10-12] The effectiveness of these treatments in stallions should be explored.

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Postpartum septic/toxic metritis in the mare – observation & rationale for treatment

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Introduction

The incidence of infection of the uterus within seven to ten days postpartum (i.e., postpartum metritis; sometimes involving the endometrium, myometrium and perimetrium) in foaling mares is low, but increases when birthing trauma and/or retained placenta occurs. Mares having an apparently normal parturition can also develop postpartum metritis. Sequelae of postpartum metritis, sometimes life-threatening, vary from delay in uterine involution to development of septicemia/toxemia, laminitis, and death.[1-6] Prompt recognition and treatment of metritis can prevent sequelae from occurring, and will more rapidly restore uterine health to normal with resultant fertile condition.

Keywords: Metritis, postpartum infection, uterus, mare

Occurrence and clinical signs

Septic/toxic metritis may occur with or without dystocia, obvious trauma to the birth canal, or retained placenta, and becomes apparent within one to ten days (most commonly on days 2-4) postpartum. The uterine atony or inertia that is purported to accompany retained placenta has been postulated to be involved in the etiology of septic/toxic metritis.[3] Trauma to the uterus, autolysis of placental remnants, and excessive accumulation of lochia are thought to contribute to rapid growth of bacteria with toxins produced that may be absorbed into the blood stream, particularly when expulsion of contents is delayed or when the normally intact uterine mucosal barrier is damaged.

The pathogenesis of toxic metritis, and particularly the time sequence in development of toxemia, remains hypothetical. Interestingly, infusion of potentially lethal doses of *Escherichia coli* endotoxin into the uterus of normal foaling pony mares on days 1 and 4 postpartum failed to result in detectable presence of endotoxin within the blood (using the Limulus amoebocyte lysate [LAL] assay for endotoxin detection), and the neutropenia that typically accompanies experimentally-induced endotoxemia did not occur.[7] Evaluation of scanning electron micrographs in that study revealed the endometrial mucosa of both treated and control mares was intact, whereas the uterine mucosal lining of one mare with clinically-apparent septic/toxic metritis that died was markedly disrupted.[8] Apart from the timing of development of septic/toxic metritis and/or laminitis, when the uterus is damaged may be conjectural; however, it is logical to assume a damaged mucosal barrier allows absorption of locally produced toxins. Septic/toxic metritis with neutropenia was found to be more common in mares that retain their placenta, particularly after dystocia.[5] Certainly, disruption of the endometrial mucosal barrier is a risk of dystocia, or disruption could gradually progress after retention of fetal membranes that leads to bacterial overgrowth. When intraperitoneal injections of *Escherichia coli* endotoxin are given, pronounced neutropeia occurs rapidly (i.e., within 1-2 hours usually), while the pronounced neutropenia with degenerative left shift seen in those mares that develop septic/toxic metritis occurs on days 2-4 postpartum.[5,8,9] Thus, perhaps a gradual buildup of endotoxin producing bacteria within the uterine lumen concurrent with progressing damage to the endometrial mucosa allows ever more absorption of endotoxin into tissues and the bloodstream.

If a foaling mare did not have a difficult birth and expelled fetal membranes were not examined for completeness, the first clinical signs in a mare developing septic/toxic metritis may be inappetance, depression, and fever. In some cases, the veterinarian may not be alerted to the potential disorder until he/she is requested to examine a postpartum mare for lameness.[3,6] Querying the owner about behavior of the mare's foal may reveal it has been noted to suckle often, yet not be satisfied, due to decreased milk production by the dam. The mare's heart and respiratory rates are often elevated when fever is present. The mare's mucous membranes may be tacky (owing to dehydration) and, if toxemia exists, the membranes may be discolored with poor perfusion (prolonged capillary refill time). A copious, fetid vulvar discharge is often evident, particularly in those mares affected by trauma and necrosis of the birth

canal. Repeated abdominal straining sometimes occurs when vaginal necrosis or lacerations are present, or if placental remnants are lying in the birth canal. The mare's feet should always be examined for warmth, presence of increased digital pulses, and pain. If laminitis develops, lameness (particularly of the front feet) can become pronounced. Sinking of the digit(s) and rotation of the third phalanx(ges) may follow, necessitating euthanasia.

Examination of the uterus per rectum and by transrectal/transabdominal ultrasound commonly reveals an enlarged atonic uterus containing an excessive amount of variably echogenic fluid. Aseptic manual examination of the birth canal is recommended to determine if any accompanying swelling, hemorrhage or necrotic tissue are present in the birth canal, which would confirm birthing trauma. Manual examination of the uterine lumen should be performed to detect the presence of placental remnant(s), including a retained tip of the placenta that previously occupied a uterine horn.

Additional diagnostic tests

A complete blood count is useful for confirming if sepsis or toxemia has developed, as neutropenia with a degenerative left shift is commonly found. Neutrophils sometimes are found to be 'toxic', evidenced by hypersegmented nuclei and cytoplasmic vacuolation.[5,10] In one study involving foaling mares referred to a hospital for dystocia, neutropenia was most pronounced 3-4 days postpartum. Absolute neutropenia (ie, often < 1500 neutrophils/μL) has been described as a common finding in mares with septic/toxic metritis.[5,9] While the relationship between bacterial endotoxemia and onset of laminitis is currently questioned, signs of endotoxemia have been linked to significant risk of laminitis developing.[10]

Swabbing the uterine lining and retained lochia for bacterial culture has been recommended to identify the organism(s) present, and to guide selection of antimicrobial(s) used in treatment of infection. Bacteria typically isolated from the uterus of mares with metritis after correction of dystocia include aerophilic and microaerophilic or anaerobic organisms – such as *Escherichia coli* (a potent endotoxin-producer) and *Streptococcus* spp., and sometimes *Bacteroides fragilis* and *Clostridium* spp.[3,5]

When foot soreness develops, radiographs should be procured and evaluated for sinking or rotation of the digit. When sinking of the digit is suspected due to 'ledging' being evident at the coronary band, contrast material can be infused into the vasculature to evaluate the circulatory status of the foot. Once vascular disruption has occurred, the prognosis for survival is grave.

Treatment

Various treatments for postpartum metritis in mares have been advocated. Systemic administration of broad spectrum antibiotics is indicated to control infection. Antibiotics selected should be effective against both gram positive and gram negative bacteria. Administration of gentamycin (6.6 mg/kg, q24h, iv) and penicillin (22,000-44,000 units/kg Na^+ or K^+ penicillin, q6h, iv or im; or 22,000 units/kg procaine penicillin q12h, im) can be used for this purpose. If endotoxemia is considered a significant risk, administration of polymixin-B (1,000–6,000 units/kg q6-8h, slowly iv) may be added to the systemic antimicrobial regimen.[11] Pentoxyphylline (7.5-10 mg/kg, q 8-12 h po or iv) may provide added benefit to improve malleability of erythrocytes and thus potentially improve circulation to vasculature of the foot.[11] For additional protection against endotoxemia, and for anti-inflammatory effects flunixin meglumine (0.25 mg/kg, q8h, iv) may administered. Specific treatment of the genital tract includes oxytocin therapy (10-20 IU oxytocin q6h, im), stimulating uterine contractions (lasting 20-50 minutes) that aid in expulsion of uterine contents. Uterine lavage can be performed to remove debris and bacteria from the uterus, thereby reducing contamination and creating a less favorable environment for bacterial growth. Uterine lavage prior to antimicrobial treatment will also remove purulent material and cellular debris that bind to and inactivate many antibiotics. However, whether there is additional benefit to administration of local antibiotics by uterine infusion is controversial. There may be some benefit to controlled exercise if foot soreness is not present, but exercise is considered contraindicated if foot soreness is present.

For uterine lavage in postpartum mares, the author prefers to utilize a large bore tube (eg, stomach tube). After disinfection of the perineal and vulvar areas, a sterilized or disinfected nasogastric tube is passed into the uterus. The hand must be cupped around the end of the tube to prevent the uterus and any remaining placenta from being siphoned into its end. Gentle lavage with warm (40°C to 42°C), sterile, physiologic saline (administered via a sterile or disinfected stomach pump) in three- to six-liter amounts can be performed until the effluent is relatively clear.[6] If necessary, uterine lavage can be repeated on successive days until uterine infection is controlled.

Acute laminitis is a medical emergency, and treatment should commence as soon as possible. Many therapeutic regimens have been recommended for the treatment of laminitis. Current equine medicine textbooks should be consulted for discussion of various treatments that may be indicated. Certainly, padding of the foot (frog), administration of anti-inflammatory/analgesic medications, and restriction of movement are indicated as part of the treatment regimen.

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Treatment of placentitis: where are we now?

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Placentitis continues to represent a significant cause of pregnancy loss for the mare. Placentitis is most commonly caused by bacteria ascending through the vagina.[1,2] *Streptococcus equi* subsp. *zooepidemicus (S. zooepidemicus)* is the bacteria most frequently isolated from clinical cases of placentitis.[1,3] Placental pathology is generally localized to the area of the cervical star with thickening and separation of the chorioallantois from the endometrium.[1,3,4] Affected mares usually have a vulvar discharge, develop an udder, and deliver a premature, compromised or dead foal. In chronic placentitis, a foal may be born precociously mature for gestational age if premature labor is delayed.[5] Therefore, the treatment goal in mares with placentitis is to prolong gestation long enough to allow fetal maturation and delivery of viable neonate.

Information from a well-established model of placentitis has directed treatment approaches.[6-10] While bacterial infection is thought to initiate disease, secondary inflammation and prostaglandin production are likely culprits in premature delivery of foals. Mares with induced placentitis showed higher concentrations of IL-6 and IL-8 in their placentas, elevated concentrations of prostaglandins E_2 and $F_2\alpha$ (PGE_2 and $PGF_2\alpha$) in allantoic fluid and increased duration and intensity of uterine contractions when compared to uninfected, control mares.[6,7,11] Histopathologic findings from the placentitis model revealed bacteria on the chorionic surface, allantoic and umbilical inflammation, and bacterial colonization in fetal lungs.[4] It is postulated that fetal infection is established by passage of bacteria through fetal membranes and into amniotic fluid that the fetus inhales or swallows. Therefore, it is likely that both infection and inflammation are important in placentitis-induced preterm delivery. As such, treatment approaches are directed at bacterial eradication, control of inflammation and amelioration of uterine contractions.

Several therapeutic agents that are commonly administered in clinical practice (antimicrobials, anti-inflammatories and progestins) have been tested in mares with experimentally-induced placentitis. Placental drug transfer in mares with placentitis was investigated using a novel *in vivo* microdialysis system to measure drug concentrations in allantoic fluid after administration.[12,13] Penicillin and trimethoprim sulfamethoxazole (TMS) achieved minimum inhibitory concentration (MIC) against *S. zooepidemicus* in allantoic fluid of mares with induced placentitis, while gentamicin was detectable at concentrations effective to treat *Escherichia coli* and *Klebsiella pneumoniae* (also implicated in placentitis). Drugs were present for up to four hours in allantoic fluid but at concentrations lower than those in serum. Pentoxifylline was detected in allantoic fluid of experimentally-infected mares, but flunixin meglumine was not. The highly protein-bound nature of flunixin meglumine likely prevented passage of this drug through the microdialysis membrane, thus rendering results regarding placental passage of the drug inconclusive. However, flunixin meglumine is still used as an anti-inflammatory drug of choice in clinical cases of placentitis.

Foal viability after mares were treated with a variety of drug combinations has also been assessed. Long term administration of TMS and pentoxifylline tended ($P = 0.07$)[14] to extend gestational length in mares with placentitis when compared to infected, untreated mares. However, foal survival was not improved in treated animals (one live foal in each group). Interestingly, TMS and pentoxifylline were present in fetal and placental tissues. So, while TMS and pentoxifylline show good penetration of placental and fetal tissues, this drug combination was insufficient to prevent preterm delivery in this study.

Progestins (altrenogest; Regu-Mate®, Intervet/Schering-Plough Animal Health, Summit, NJ) have also been combined with TMS and pentoxifylline to treat mares with induced placentitis.[15] Progestins are postulated to promote uterine quiescence through reduction in myometrial gap junctions and oxytocin receptors.[16,17] Administration of progestins in women with high risk pregnancies has shown to reduce the incidence of preterm labor, and this treatment has become standard practice.[18] When mares with induced placentitis were administered TMS, pentoxifylline and altrenogest, 10 of 12 (83%) delivered viable

foals.[15] All five untreated, infected mares aborted or delivered non-viable foals. Most live foals had negative blood cultures at birth and normal parameters for complete blood count, serum chemistry, cortisol and IgG. It was concluded, from these data, that mares with placentitis benefited from treatment using an antimicrobial, anti-inflammatory agent and progestin. The authors of this study were careful to note that early initiation of treatment after experimental infection (within 96 hours) likely contributed to the high number of live foals in this study.[15]

Recently, workers from Mississippi used an evidence-based approach to evaluate different treatment protocols in mares with induced placentitis. Mares were administered TMS, alone, or combined with anti-inflammatory agents (dexamathasone and aspirin), and/or with progestins (altrenogest + aspirin).[19] Interestingly, mares administered TMS, alone, were as likely to deliver viable foals (4/6; 63%) as mares administered TMS in combination with dexamethasone, aspirin and altrenogest (13/18; 72%). These data prompt the question of whether anti-inflammatory agents are important to treatment of placentitis, or whether antibiotics (TMS) are sufficient to treat the disease. Interestingly, work in non-equine species suggests that multifaceted therapy is warranted for prevention of preterm delivery. Using a non-human primate model of placentitis, workers examined the effects of anti-inflammatory agents (indomethacin[20], dexamethasone and interleukin-10[21]) to stop preterm delivery. In all experiments, treated monkeys had lower amniotic fluid prostaglandin concentrations and uterine contractions than untreated controls. None of the agents effectively inhibited production of pro-inflammatory cytokines. Administration of dexamethasone prevented preterm delivery of fetuses. The effect of antibiotic, alone, was also tested in the primate model. Monkeys inoculated with group B streptococci (in the amniotic cavity) were administered ampicillin, alone, or in combination with dexamethasone and indomethacin.[22] Ampicillin effectively eradicated bacteria from the amniotic fluid of infected animals. However, amniotic fluid cytokines, prostaglandins and uterine contractions persisted in the face of maternal antibiotic treatment. When dexamethasone and indomethacin were added to ampicillin, cytokines and prostaglandins were suppressed as were uterine contractions. From these studies, one can speculate whether antimicrobials, alone, are more effective early after infection and anti-inflammatory treatment becomes important in more chronic disease. Unfortunately, in a practical setting, it is often difficult to predict the onset of placentitis and treatment must be initiated quickly. In these cases, it can be difficult to select a conservative treatment approach.

Limitations in antimicrobial choices are also problematic for the practitioner treating mares with placentitis. Oral administration of drugs is ideal in a field setting. However, TMS-based therapy does not consistently result in delivery of a live foal. Additionally, over 50% of uterine cultures obtained immediately postpartum from mares with induced placentitis were positive for *S. zooepidemicus* despite prolonged administration of TMS.[15] This is in contrast to negative uterine cultures obtained from normal foaling mares.[23] Studies have shown that TMS is not consistently effective in eradicating *S. zooepidemicus, in vivo*, despite *in vitro* susceptibility of pathogens and high concentrations of TMS at the site of infection.[24] However, few alternative oral preparations of antimicrobials are available, as are parenterally administered drug choices that can be used in field conditions.

Ceftiofur, a third generation cephalosporin, has excellent bactericidal activity against streptococcal organisms as well as many gram negative aerobes and some anaerobes.[25-27] Ceftiofur penetrates body fluids, the endometrium, joints and pulmonary sites of infection with concentrations that equal or rival ampicillin or potentiated sulfonamides.[28,29] Ceftiofur sodium, marketed as Naxcel®, is a commonly used antimicrobial in equine practice. While not as convenient as orally administered TMS, once daily, intramuscular injection of ceftiofur sodium provides a practical method of administering a parenteral drug. However, the effectiveness of ceftiofur sodium for treating mares with placentitis is unknown.

In 2010 Pfizer Animal Health (New York, NY) received Federal Drug Administration approval for the use of long-acting ceftiofur crystalline free acid (CCFA; Excede®) for treatment of horses. Excede® has broad appeal for the equine practitioner because it provides therapeutic drug levels in horses for 10 days when administered at four day intervals. Additionally, CCFA is a potent antimicrobial against *S. zooepidemicus*. In many ways, this exciting new drug would appear to be the perfect

antimicrobial treatment for mares with placentitis. Consequently, Excede® was recently tested in mares to determine the ability of the drug to penetrate fetal membranes and the effectiveness of the drug for preventing abortion in mares with induced placentitis. Mares were administered Excede®, alone (n=3) or in combination with pentoxifylline and altrenogest (n=6). Three mares served as infected, untreated controls. Concentrations of ceftiofur metabolites were measured as an indicator of drug distribution in mares, foals and placental tissues using high performance liquid chromatography (HPLC; University of CA Davis).

Serum concentrations of ceftiofur metabolites in mares were consistent with expected profiles after administration of this drug.* However, drug concentrations measured in fetal and placental tissues were considerably below therapeutic concentrations indicating low penetration of ceftiofur metabolites across fetal membranes. Further, foal survival was poor after treatment with this drug (live foals = 0/3 after Excede® alone, and 2/6 after combination treatment). Bacterial eradication using this antibiotic was not achieved in uterine or foal samples from animals with confirmed bacterial infections after inoculation. It is likely that much higher serum concentrations of ceftiofur metabolites would be necessary in order to achieve therapeutic concentrations in placental and fetal tissue. However, since, Excede® is slowly released from the site of injection it does not tend to provide very high serum concentrations even with higher dose administrations. Therefore, ongoing work is investigating the ability of ceftiofur sodium to pass through fetal membranes and attain therapeutic concentrations in fetal fluids and tissues.

In summary, conscientious treatment of equine placentitis is challenging. Data regarding treatment are sometimes conflicting, and results after treatment in a clinical setting can be disappointing. Yet, salvaging a pregnancy can be enormously rewarding. Ongoing efforts by several investigators are focusing on earlier diagnostic methods, allowing for more rapid initiation of treatment, and hopefully, more consistent effects of treatment.

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Biopsy and vitrification of equine expanded blastocysts

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Abstract

Preimplantation genetic diagnosis and embryo cryopreservation have extensive clinical application in the horse. Biopsy of equine embryos is complicated by the presence of the equine embryonic capsule. Recently, we developed a technique to puncture the capsule and obtain cells from the trophoblast of expanded equine blastocysts, using micromanipulation with the Piezo drill. Biopsies were obtained without affecting the pregnancy rate of the blastocysts after transfer. Genetic analysis after whole genome amplification of the biopsied cells demonstrated some failure to detect signal, and allele dropout, and work is currently underway to improve the accuracy of genetic analysis. Observation that the blastocysts collapsed after biopsy, yet maintained viability, led to the hypothesis that the collapsed blastocyst might be successfully vitrified. Further work showed that vitrification of collapsed blastocysts using an ethylene glycol-containing medium was associated with high pregnancy rates after transfer (71% of embryos produced pregnancies that developed normally to the heartbeat stage), thus providing the first successful procedure for cryopreservation of expanded equine blastocysts. These advances in embryo manipulation techniques should find wide application in clinical equine practice.

Keywords: Embryo, cryopreservation, horse, preimplantation genetic diagnosis

Biopsy of embryos for preimplantation genetic diagnosis

Devastating genetic diseases such as hereditary equine regional dermal asthenia (HERDA), glycogen branching enzyme deficiency, hyperkalemic periodic paralysis (HYPP), polysaccharide storage myopathy, severe combined immunodeficiency disorder, and cerebellar abiotrophy have been identified in the horse.[1-3] With the exception of polysaccharide storage myopathy, all of these diseases are inherited in a recessive manner. This, and the fact that some of the top-performing horses in their breeds carry these recessive mutations, make it difficult to eliminate the causative alleles from the population.

Preimplantation genetic diagnosis (PGD) via embryo biopsy is commonly utilized in humans to determine the genetic status of at-risk embryos.[4] In humans, to perform PGD, embryos must be produced in vitro. In the horse, while in vitro embryo production is possible, it is performed at only a few centers, is expensive, and is inefficient relative to in vivo embryo collection. Thus, for equine PGD to be a viable clinical procedure, it must be performed on embryos recovered from mares after uterine flush. However, the presence of the equine embryonic capsule, which starts to develop as soon as the embryo enters the uterus, on late Day 5 after ovulation,[5,6] may complicate both the performance of the biopsy and the survival of the embryo after biopsy.[7,8]

Using unexpanded (e.g., Day-6) embryos for PGD avoids the presence of the capsule, and several laboratories have reported successful biopsy of early (Day-6 to 7.0) embryos.[7-9] Transfer of the biopsied embryos resulted in pregnancy rates of 21 to 75%. The cells recovered on biopsy were successfully analyzed for genetic sex in these studies. However, biopsy of larger embryos was associated with lower pregnancy rates (29%).[7] One hypothesis that might be made from these data is that younger (e.g. Day-6) equine embryos can repair damage to the capsule, whereas older, fully encapsulated embryos, cannot. This hypothesis is supported by the finding that pregnancy rates after embryo bisection, in which the capsular material and zona pellucida are lost, were 23 to 67% for embryos bisected at the morula or early blastocyst stages, but 0/12 for embryos bisected at the expanding blastocyst stage.[10,11]

Unfortunately, most clinicians do not perform equine embryo recovery before Day 7, as the small size of the earlier embryo makes the embryo harder to locate, thus increasing search time, and recovery rates have been reported to be lower when uterine flush is performed on Day 6 than on later days.[12-14] For clinical application of PGD, it is important to determine whether it is possible to obtain biopsy samples from Day-7 embryos, having fully formed capsules, and if so, whether these embryos can maintain

viability after biopsy. In addition, to apply PGD clinically, embryos must be shipped to the laboratory from the field. Equine embryos are commonly shipped overnight to embryo transfer facilities in passive heat exchange devices (Equitainer®, Hamilton Research, Inc., South Hamilton, MA), and thus it is important to examine the effect of overnight shipping on the survival of embryos after subsequent biopsy.

We performed studies to determine if embryo biopsy using micromanipulation could be performed on Day-6 and Day-7 equine embryos, and if this method was effective in recovering sufficient cells for genetic analysis.[15] Biopsy was performed by micromanipulation, using a Piezo drill to penetrate the zona and/or capsule, then cells were aspirated from the trophoblast layer through the manipulation pipette. We began by performing biopsies on Day-6 embryos. Pregnancy rates for these embryos, which had intact zonae pellucidae at the time of recovery, were 3/3 for those biopsied immediately after recovery, and 0/3, 1/3 and 2/3 for embryos shipped overnight at cold, room temperature, and warm temperatures, respectively. We utilized shipping warm for subsequent studies. We then evaluated the effect of biopsy on expanded, encapsulated blastocysts. Pregnancy rates for Day-7 expanded blastocysts were 5/6 for those biopsied immediately after recovery, and 5/6 for those biopsied after being shipped overnight warm. All pregnancies developed normally to the heartbeat stage. We also biopsied four Day-8 blastocysts; two, 790 and 1350 μm in diameter, established normal pregnancies (to the heartbeat stage) after biopsy. Nine mares carrying Day-6 and Day-7 biopsied embryos were allowed to maintain pregnancy, resulting in birth of nine normal foals. From these findings, we concluded that biopsy of expanded equine blastocysts with the Piezo drill is possible and does not compromise embryo viability.

Genetic analysis of the biopsied cells met with some problems. We saved the biopsies from the nine embryos that produced foals, and then compared the genetic analysis of these cells, after whole-genome amplification, with that of the resulting foals. One embryo biopsy was lost when the vial cracked on thawing. Sex was successfully determined from amplified DNA in 8/8 embryos.

The eight embryo biopsy/foal pairs were also evaluated for HYPP status (*SCN4A* gene) and for HERDA status (*PPIB* gene). In evaluating the accuracy of detection of allele status, the major problem associated with analysis of small samples, such as embryo biopsies, is "allele drop-out," that is, the amplification of only one allele in an animal which is in fact heterozygous. When only one allele is detected, the sample is interpreted as being homozygous for that allele, rather than being accurately detected as heterozygous. In our embryos, the *SCN4A* gene failed to amplify altogether in two embryos, and the *PPIB* gene failed to amplify in one embryo. Analysis of the remaining embryo/foal pairs was confounded by the fact that all foals were homozygous normal for the normal *SCN4A* gene, and six of eight foals were homozygous normal for the *PPIB* gene; thus there were only two foals that were heterozygous for a disease-causing gene and could be used to test for allele drop-out. In these two samples, one was accurately detected as being heterozygous, but the other was interpreted as being homozygous for the affected allele, thus it had suffered from allele drop-out.

At the time of writing, we are exploring alternative methods of whole-genome amplification with Dr. Cecilia Peneda of the University of California at Davis Genetics Laboratory, and the results are very promising. In a recent trial of 16 biopsy samples from four embryos, one sample was lost on thawing, and one failed to amplify, but the remaining 14 samples had excellent fidelity in detection of heterozygous coat color and parentage identification loci when compared to the results obtained by analyzing the whole embryo. We will continue these studies with additional embryos that are heterozygous for important disease-causing mutations, to ensure that these loci are also detected with good fidelity.

From this study, we can conclude that the capsule of the equine embryo can be breached without impairing viability. Embryos may be collected from mares on Day 6 or -7 after ovulation, and shipped to the laboratory overnight in standard commercial embryo holding medium, biopsied, and shipped back for transfer with no detrimental effect on pregnancy rates or live foal rates. We anticipate that PGD may soon become a viable clinical procedure.

Embryo vitrification

Embryo vitrification is a relatively simple technique that may be performed in practice without extensive equipment, and has been well described previously.[16] Freezing and vitrification of early equine embryos (less than 300 μm in diameter) is effective; however, freezing or vitrification of embryos >300 μm diameter has resulted in low pregnancy rates after transfer.[17-21] Unfortunately, this has necessitated recovery of embryos on Day 6 after ovulation if cryopreservation is to be performed, which, as noted above, may be problematic.

While conducting the above studies on embryo biopsy, we realized that to apply the biopsy procedure clinically, the embryo would have to be held before transfer, while the genetic analysis of the biopsied cells was conducted. Cryopreservation seemed like the most logical method to hold the embryo. We hypothesized that while cryopreservation of expanded Day-7 blastocysts has low success, perhaps the breach in the capsule induced by the biopsy procedure would aid in cryopreservation of blastocysts after biopsy. We evaluated viability of three blastocysts that had been biopsied, allowed to reform in culture (reformation of the blastocoele occurred with 3 h), then vitrified, and compared this to the viability of a blastocyst that was vitrified immediately after biopsy, while still collapsed. Only the blastocyst that was vitrified while collapsed grew normally in culture after warming. This was surprising to us, as we thought of biopsy, collapse and vitrification as a series of insults that might be additive; however, on further consideration, it made biological sense that collapse eliminated the large volume of blastocoele fluid, which might aid the speed of vitrification, and perhaps allowed penetration of the cryoprotectant through the biopsy-induced defect in the trophoblast.

To further investigate, we conducted a study on the vitrification of embryos after blastocoele collapse.[22] We started by evaluating different methods of vitrification using non-biopsied, small embryos (<300 μm). A recent paper had reported success in vitrification of ferret embryos, which contain high amounts of lipid similar to horse embryos, using a fine-diameter microloader pipette tips.[23] We vitrified small, non-biopsied embryos using dimethylsulfoxide-containing medium (DM), as described in the paper of Sun et al. on ferrets, or using ethylene glycol-containing medium (EG), which had been used successfully in early equine embryos.[24] Both media were used in conjunction with the micropipette tips. After we realized that embryos in the EG group were not developing well after warming, we introduced a third group, EG-vitrified embryos warmed by the procedure described by Sun et al., using sucrose (EG/s). Embryos in the DM and EG/s treatments grew in culture after vitrification, and so we investigated the pregnancy rates after transcervical transfer. Both groups of embryos established pregnancies after transfer (3/12 and 3/6 for DM and EG/s respectively).

These vitrification methods were then applied to expanded blastocysts collected on Day 7 after ovulation (300-730 μm in diameter). The blastocysts were biopsied, then vitrified immediately while in the collapsed state. The blastocysts were subsequently warmed and transferred to recipient mares. Rates of normal pregnancy (detection of embryonic heartbeat) were 2/16 (13%) and 6/13 (46%) for DM and EG/s treatments, respectively. We conducted further studies using only the EG/s treatment.

The estimated percentage of blastocoele fluid lost after biopsy was recorded for the 13 embryos in the EG/s group, and this was evaluated in relationship to the pregnancy status after transfer for these embryos. The pregnancy rates were 0/3, 2/5 and 4/5 for embryos losing <10%, 20 to 30%, and 70 to 100% of their blastocoele fluid after biopsy, indicating that greater loss of blastocoele fluid after biopsy was associated with higher survival. Therefore, in the next study, we evaluated an altered ("Central") biopsy technique, in which the biopsy pipette was introduced into the center of the blastocoele and the fluid aspirated, then cells biopsied from the central area. This was followed by vitrification in EG/s. However, after thawing and transfer, pregnancy rates were only 1/8 (13%) for embryos cultured after warming and 4/7 (57%) for embryos transferred immediately after warming. This suggested that the Central technique may have been damaging the embryo, perhaps from the vigorous aspiration and suction to eliminate fluid or obtain cells from the inner surface of the blastocoele.

In our final study, therefore, we returned to biopsy of cells from the periphery of the blastocyst, but performed gentle suction to assure the removal of blastocyst fluid. Expanded blastocysts 407 to 565 μm in diameter were biopsied in this manner, then vitrified with EG/s. Transfer of these embryos after

warming resulted in a pregnancy rate of 6/7 (86%), with a rate of normal pregnancy (embryos with heartbeats) of 5/7 (71%).

These findings are exciting, demonstrating for the first time that expanded equine blastocysts can be successfully vitrified. Much further work is needed to refine the method for blastocoele collapse, hopefully to eliminate the need for micromanipulation, and to determine whether such collapsed blastocysts may be vitrified using standard techniques.

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General techniques and organization of large commercial embryo transfer programs

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Introduction

Equine embryo transfer (ET) started in Argentina in 1990 primarily in polo ponies.[1,2] This presentation is a review of the practical experience gained over the last 20 years of commercial ET in Argentina. Although the author has been involved in several ET programs since 1990, the data analyzed in this work were obtained only at Centro de Reproducción Equina Doña Pilar, located in Lincoln (B), Argentina and involves the production of 7,939 pregnancies obtained from 13,942 uterine lavages for embryo collection (flushings) between 1997 and 2010. A summary of this work is detailed in Table 1.

Table 1: Number of flushings performed at Doña Pilar 1997-2009 (n=13942 flushings)

Season	Donor mares	Stallions	Flushings	Embryos	Preg 14-21 days	Preg 60 days	Embryo rec. (%)	Pr 1 (%)	EED (%)	Efficiency (%)
97-98	56	12	213	141		69	66.2%			
98-99	50	11	231	192	133	105	83.1%	69.3%	21.05%	57.58%
99-00	58	14	294	270	149	133	91.8%	55.2%	10.74%	50.68%
00-01	76	20	326	282	189	164	86.5%	67.0%	13.23%	57.98%
01-02	96	16	357	322	243	216	90.2%	75.5%	11.11%	68.07%
02-03	128	23	487	426	325	271	87.5%	76.3%	16.62%	66.74%
03-04	188	31	964	870	558	484	90.2%	64.1%	13.26%	57.88%
04-05	275	35	962	1043	671	574	108.4%	64.3%	14.46%	69.75%
05-06	352	48	1744	1413	861	750	81.0%	60.9%	12.89%	49.37%
06-07	431	44	2287	1845	1036	889	80.7%	56.2%	14.19%	45.30%
07-08	448	55	2331	1823	1286	1162	78.2%	70.5%	9.64%	55.17%
08-09	500	53	2288	1951	1454	1278	85.3%	74.5%	12.1%	63.55%
09-10	359	44	1671	1313	1034	949	78.6%	78.8%	8.2%	61.88%

The ET industry grew tremendously during the last 10 years in Argentina. Many of the best playing mares were sold overseas at the peak of their athletic careers so their bloodlines were being lost. Embryo transfer became the solution to this problem allowing us to produce offspring before the mares were sold abroad. The technique rapidly showed its advantages and became widely accepted by polo players and breeders. This resulted in a need to rapidly adjust to the market demand. We introduced changes in many procedures to make them faster and simpler and also changes in general and reproductive management. Despite these changes the fast increase in labor caused several "crises" that affected the overall efficiency of our program. The analysis of these data helped us to identify some of the factors that affect the efficiency of an embryo transfer program.

Keywords: Embryo transfer, estrus synchronization, recipient management

Procedures in an ET program

Selection and management of donors

Most mares brought to our center are polo ponies. Many of these mares are still at the peak of their athletic careers. They are enrolled in the program for three to four months after the polo tournaments and during this time undergo intensive reproductive management to produce several pregnancies. Other donors are old retired polo pony mares that come to the program to maximize the number of pregnancies produced per season. In some instances the mares have failed to produce offspring naturally due to fertility problems.

Breeding soundness evaluation. Before admission to the center, every mare is tested for equine infectious anemia. Once the mare arrives, a general physical examination is performed. All mares are dewormed and vaccinations are updated. Records and fertility history, including previous ET records, are carefully reviewed. Each donor is accompanied by the following information upon arrival: number of pregnancies to achieve, sires to be used on each cycle, and type of housing and feeding to which the mare is accustomed. The mares are carefully identified with tags, and samples are submitted for DNA typing. Every mare undergoes a breeding soundness evaluation after arrival at the ET center. In some old mares with defective perineal conformation, surgical correction is performed before starting the program. If a fertility problem is detected, appropriate therapy is instituted.

Management of embryo donors. At Doña Pilar, embryo donors are housed in groups of 10 to 25 to optimize management and decrease stress and chances of injury. Once a mare is introduced and adapted to a social group, she will stay with that group for the rest of the season. Donor groups are assigned to one of two examination facilities directed by two different veterinarians on each team. An average of 150 donor mares are examined, inseminated, and flushed by each team.

Selection and management of recipients

One of the most critical aspects of an ET program is the selection, management, and quality of the recipient mares.[3-5] Good recipient mares should meet all of the following requirements: (1) good health and body condition, (2) easy to handle and halter broken, (3) body size similar to that of the embryo donor,[6,7] (4) 4–10 years of age, (5) sound breeding condition with a uterine biopsy grade I or IIA according to the criteria of Kenney,[8,9] and (6) regular estrous cycles. We prefer mares that have foaled normally at least once and that have shown good ability to nurse the foal. Although primiparous mares can be used, it is important to advise the owner of the embryo that the mare may need more attention at the time of foaling and that foals can be of smaller size at birth.[10] Our recipient herd consists of crossbred mares weighing between 400 and 600 kg. Health requirements for recipient mares are the same as those for donor mares. In addition, all recipient mares are freeze branded. Careful records include identification information, age, markings, vaccination status, deworming status, and reproductive history, if available. The breeding soundness examination for the recipient mare is similar to that performed on the donor mare.[8] Special emphasis is given to the size and tone of the uterus and cervix. We prefer to use recipient mares with documented, well-known reproductive histories.

Recipient mares are kept in mixed pastures of grass and alfalfa. Pregnant and transferred recipients receive the best pastures, especially from the day of transfer up to 40 days of gestation. Non-pregnant mares are kept in groups of approximately 50 to 100. These groups are examined periodically depending upon the synchronization requirements to determine follicular activity. Time of ovulation is determined within a range or "window of synchrony" as described below.

Recipient management. Recipient management to avoid stress is a critical factor that affects pregnancy rates in a large-scale ET program.[11-13] It is very common for recipient mares added to the program in the last trimester of the breeding season not to become pregnant and go into anestrus earlier than the rest of the group. The ability to overcome this problem is one of the major challenges in a large commercial program.

Synchronization

Synchronization between the estrous cycles of donor and recipient mares is the most time-consuming activity in an ET center. Mares are routinely examined by transrectal palpation and ultrasonography of the ovaries and internal genitalia.[8,14] Donor mares in estrus should be examined periodically once a dominant follicle has been detected. This is essential for deciding the time for artificial insemination and for determining the day of ovulation (day 0). At Doña Pilar we use recipients that ovulate from the same day as the donor (synchrony 0) up to four days after the donor (sync +4). Synchronization between donors and recipients is better understood if the days of progesterone influence of the recipient uterus at the time the embryo transfer are considered. We

prefer recipients to have been under progesterone influence for at least four and not more than seven days at the time of transfer. The progesterone influence can be from endogenous progesterone if the recipient has effectively ovulated or from exogenous progesterone in those cases when the recipient is not cycling. An artificial estrous cycle in the recipient can be produced by means of injections of estrogens and progesterone and be effectively used as it is described later in this work.

The method used for synchronization depends upon the number of donors and recipients involved in the program. If there are a large number of recipients available, synchronization may be performed by administration of a luteolytic dose of prostaglandin $F_2\alpha$ (PGF) or an analog given to one or two recipients one or two days after administration to the donor.[15,16] In large programs recipient availability can sometimes be a limitation. The use of ovulation-inducing agents such as human chorionic gonadotropin (hCG) and deslorelin acetate is common in ET programs.[17,18] Injection of 1500 IU of hCG intravenously when there is a 35 mm follicle induces ovulation 36–48 hours after injection. Deslorelin acetate (1.5 mg sc) is also commonly used at Doña Pilar to tighten the synchrony between donors and recipients.

The use of intact non-cycling mares supplemented with progesterone to mimic a regular estrous cycle has been a useful alternative when recipients stop cycling at the end of breeding season.[19-22] As previously mentioned, it is common that new mares added to the program at the end of the season enter anestrus. In these cases we administer estradiol benzoate (2 mg/day for two to three days) and then 300 mg of progesterone daily for four to five days before using the mare as a recipient.[23] On the day of ET the anovulatory mares are treated with progesterone in oil (300 mg im) plus biorelease progesterone (1.8 gm im). The biorelease progesterone (P4LA) treatment is repeated on a weekly basis thereafter, until day 110 of gestation. Progesterone administration could be discontinued once secondary corpora lutea are detected. Some ET programs use altrenogest instead of progesterone. Altrenogest does not cross react with progesterone so endogenous progesterone produced by secondary corpora lutea can be determined which indicates if altrenogest administration can be safely discontinued.

In a study conducted at our clinic, a total of 469 transfers were performed between February 15 and April 30, 2008 and pregnancy rates achieved in normal cycling mares vs. intact non-cycling mares supplemented with progesterone were compared. The results in both groups were analyzed in 15-day periods. Overall, pregnancy rates in anovulatory, progesterone-treated recipients were significantly lower than those for ovulatory recipients (164/192 [56.1%] vs. 197/277 [71.1%]). This finding was probably related to the season in which this study was conducted (early fall); mares that become anovulatory early in the fall are typically those with lower body condition scores. Later in the anovulatory season, many mares, regardless of body condition score, will be in transition or anestrous and the pregnancy rates obtained in both groups are similar. However, the use of noncycling progesterone-treated mares is still a viable alternative to extend the ET season by one or two months.

In a retrospective analysis of our records over two consecutive breeding seasons we studied the effect of number of days of progesterone supplementation on pregnancy rates achieved at day 14-21 days after donor ovulation (Table 2). In addition, we studied the effect on early embryonic death (EED) rates. A total of 551 non-cycling hormonally treated mares were used as embryo recipients after being supplemented with 300 mg of progesterone in oil (P4) daily for five to eight days before transfer. The pregnancy rates achieved appeared to be different in mares that were treated for five, six, seven or eight days (70.45; 57.71: 56.11 and 44.44%, respectively). Early embryonic death appeared to be different among groups (9.68; 12.93; 27.42; 5.00%) for mares that received P4 for five, six, seven and eight days, respectively. Mares that received P4 for seven days experienced a much higher incidence of EED.

Table 2. P4 Supplementation (2007/2008 and 2008/2009 combined) n=551

Days of P4	Total embryos	Pr1	(%)	Pr2	(%)	EED	(%)
5	44	31	70.45	28	63.64	3.00	968
6	201	116	57.71	101	50.25	15.00	12.93
7	221	124	56.11	90	40.72	34.00	27.42
8	45	20	44.44	19	42.22	1.00	5.00

Artificial insemination

Mares are artificially inseminated with fresh, extended semen collected at the center. Mares that ovulate within 24–48 hours after breeding are not rebred if the semen is of good quality and sperm longevity is adequate. Mares that are susceptible to endometritis are inseminated using minimal contamination breeding procedures.[25] Due to the large number of donors enrolled in our program we intended to decrease the number of examinations per mare to allow us to better organize the work. We analyzed the effect on the efficiency of our program if ovulation was detected in a range of 48 hrs instead of 24.[24] Donors received either hCG (1600 IU iv) or deslorelin acetate (1.5 mg sc) when a 35mm follicle was detected with other signs of estrus (uterine edema). Donors were artificially inseminated on the same day or the day after administration of the inducing agent, depending upon semen availability and then were re-examined 48 hrs later to detect ovulation. Flushings were performed eight days after detection of ovulation. The embryo recovery rate (679/1079 [63%] vs 127/181 [70%]) was not different between these two groups. This has been a major change in our donor management because it has helped us to better organize the work and to decrease workloads on Sundays. The fact that the embryo recovery rate was higher in the group of mares examined every 48 hrs reflects the fact that difficult mares susceptible to endometritis or mares bred to stallions of short sperm longevity were all examined in a daily basis to be managed using minimal contamination techniques,[25] whereas "normal" donors with a good reproductive history bred to stallions with good semen quality were examined every 48 hrs.

Embryo collection and processing

The embryo enters the uterus from 5 days 10 hours to 5 days 22 hours after ovulation.[26,27] The possible reasons for this variability could be the delay between ovulation and fertilization, embryonic factors related to timing of prostaglandin E_2 secretion, sex of the embryo, or other individual factors. Flushings are usually performed between days six and eight after ovulation.[28-30] Recovery rate is lower when performed at day six. This can be due to one or more of the following reasons: (1) failure of the embryo to descend into the uterus by day six, (2) failure of the technician to recover the embryo from the uterus because of a higher gravity weight of the embryo, (3) failure of the technician to find the embryo because of its smaller size, and (4) loss of the embryo at some point during the process. We prefer to attempt embryo recovery on day eight after ovulation. At this stage, embryos are large enough to be easily found even with the naked eye, so chances of missing or losing the embryo are decreased.

Flushing technique. The uterine lavage, or uterine flush, is a simple procedure performed with the mare restrained in stocks. Before the flushing, the mare's rectum is evacuated of feces, and the size and tone of the uterus and cervix are evaluated. In addition, follicular status of the ovaries is established to determine whether the mare will receive PGF immediately after the uterine flush. When the mare has a follicle >35 mm, PGF treatment is delayed or a half dose is given to prevent premature ovulation. This allows the uterus to recover from the uterine flushing and increases the chances of a normal subsequent heat before re-insemination. Tranquilization is usually not necessary, but some mares may require light sedation with 50–100 mg of xylazine intravenously. Acepromazine maleate can also be used for this purpose, but we prefer not to use it because it induces relaxation of the uterus, making it more difficult to recover the fluid in some instances. Once the mare has been

examined, her tail is wrapped and hung in a vertical position. The perineal area is carefully washed with soap, rinsed with tap water, and dried with a clean paper towel. A small piece of wet cotton is used to clean the vestibule. Flushings are performed with lactated Ringer's solution with the addition of 0.5% fetal calf serum. There are several brands of flushing medium on the market. We prefer to use lactated Ringer's solution because of its lower cost and easy availability. Fetal calf serum prevents the embryo from sticking to the tubing and filter although the use of fetal calf serum is controversial. We believe that fetal calf serum facilitates embryo handling but probably does not make any difference on embryo survival or recovery rate.

The technician, wearing a sterile sleeve with a small amount of lubricating jelly on the dorsal part of the hand, introduces the arm into the vagina to identify the external os of the cervix. With the index finger, the technician dilates the external os to a size large enough to pass the tip of a 24-gauge Foley catheter into the body of the uterus. The cuff is inflated with 30 ml of air, and the catheter is pulled back gently, forming a tight seal at the internal cervical os. A total of 2–3 L of flushing medium is infused by gravity flow in aliquots of 500–1000 ml, depending on the size of the uterus. The uterus should not be overfilled which will produce discomfort for the mare and also can result in fluid lost through the cervix. The flushing catheter is connected with a Y junction to the delivery tubing on one end and to a large-volume filter on the other end. The system should be completely purged with flushing medium to eliminate the air before the procedure is started. This prevents the formation of foam and bubbles. Several brands of catheters can be used. Integrity of the air cuff should be checked before the catheters are introduced into the mare. The fluid is passed through the filter connected in line with the catheter by means of the Y junction and silicone tubing. The amount of fluid recovered is measured in a graduated receptacle and should be more than 95% of the volume infused into the uterus. In some cases, especially in old mares with a large pendulous uterus, fluid recovery can be difficult. In such cases, the use of 20 IU of oxytocin iv during the flushing procedure can aid in the recovery of the flushing medium. The use of ultrasonography to locate pockets of fluid can be helpful during the subsequent manipulation of the catheter toward these areas. Gentle massage of the uterus is performed to ensure that the medium has reached the entire uterus. This also produces a slight turbulence to get the embryo in suspension, thereby increasing the chances of recovery.

Handling and evaluation of the embryo. After the uterine flush is completed, the filter is drained so that approximately 20 ml of fluid are left in the filter. This content is swirled gently to prevent the embryo from sticking to the filter walls, and then it is poured into a sterile Petri dish. The filter is then rinsed with flushing medium to ensure that the embryo is not lost in the filter. Many of the expanded blastocysts recovered at day seven and almost all embryos recovered at day eight can be found with the naked eye or with a small magnifying glass if the effluent is clear. Consequently, the embryo can be easily found and transferred within a short period of time. If we fail to find the embryo with the naked eye, we search with a dissecting microscope first at a lower magnification and then with a higher magnification to grade the embryo quality. Embryo searching is facilitated when bubbles or foam are not present in the Petri dish. Once the embryo is found, it is rinsed at least three or four times and then transferred to a small Falcon dish containing holding medium (ViGro or SYNGRO™, Bioniche Animal Health, Athens, GA) by means of a 10 to 20 µl Unopette adapted to a 1 ml syringe. Large embryos will be handled with a 0.25-ml sterile straw since they are too large to be loaded in a Unopette. Depending upon the diameter embryos should be transferred using other devices. Embryos can be kept at room temperature in holding medium for two or three hours before transfer. If transfer is delayed, we usually cool them to 18°C.

Embryo biopsy for gender diagnosis. Embryo biopsy samples can be obtained from day seven or eight embryos for PCR determination of genetic diseases as well as gender diagnosis.[31] At Doña Pilar embryos are micromanipulated and a few cells from the trophoblast are obtained and subsequently processed for PCR analysis. Embryos are only transferred if the gender corresponds to the one requested by the owner. This service has been recently started at Doña Pilar. In a limited trial we performed biopsies on 36 embryos that were immediately transferred. Thirty-one mares were detected pregnant (86.11%) indicating that this is a promising technique that will be applied commercially in a larger scale in future ET seasons.

Embryo transfer

Selection of the transfer device. There several disposable instruments on the market developed to transfer equine embryos. It is essential to choose the appropriate instrument depending upon the size of the embryo to be transferred. Embryos collected on day eight or later may be too large to be transferred in a 0.5-ml straw in those instruments in which the opening is smaller than the 0.5-ml straw diameter. In some instances, the embryo can fit into the straw but it is larger in diameter than the opening of the transfer gun, which means that the embryo is destroyed when it is transferred. If the embryo is too large to be transferred in a conventional transfer gun, we use an AI pipette[32] or even the outer part of a 0.25-ml disposable transfer gun.[33]

Preparation for transfer. In preparation for ET we follow these steps:

1. Select the recipient to be used from among the ones available: The records of the recipients available should be carefully reviewed. It is important to note if the mare has received an embryo in the same season and did not get pregnant, or if the mare was pregnant and suffered EED. It is important to note if there were any signs of uterine abnormality such as presence of fluid or any evidence of endometritis. The recipients are evaluated before transfer and notes are taken about tone of the uterus and cervix and the presence of a corpus luteum.
2. Prepare the recipient carefully; the mare is restrained in stocks and prepared as for any other intrauterine procedure. The mare should be properly restrained at the time of transfer. If sedation is required we like to use 50–100 mg xylazine iv. In rare cases we may use detomidine at a dose of 1 mg iv given before transfer.
3. The embryo is aseptically aspirated into a 0.5-ml straw. The embryo is aspirated between two columns of medium separated by air as has been described.[1,29.] An assistant separates the mare's vulvar lips and the operator introduces his/her sterile gloved hand into the vagina The external os of the cervix is located with the index finger. Care should be taken not to dilate the cervix in this procedure, which in our experience will lower pregnancy rates. The index finger should be used just to find the external os but not to dilate it. A sanitary protector is used to pass through the vagina and halfway into the cervix. The anterior end of the transfer gun is introduced gently halfway into the cervix where the sanitary sleeve is punctured by the gun. The operator removes his/her hand from the mare's vagina and introduces it into the rectum. Gentle manipulation of the body and right horn of the uterus through the rectal wall is performed to position the transfer gun deep into the uterus. It is essential to avoid scratching the endometrium, which would induce prostaglandin release and could cause pregnancy failure. The embryo is transferred by gentle pressure to the transfer gun plunger. At our clinic most clinicians use their left hand for all gynecological procedures making it easier to transfer the embryo into the right horn. I do not think it makes a difference if the embryo is transfered deep in the body of the uterus or in the uterine horn, but in any case it is essential to avoid scratching the endometrium at the time of transfer.

Recipient mares are given a dose of long acting progesterone at the time of transfer. Although it has not been yet proven that it makes a difference, we do this in a regular basis. The author personally believes that progesterone supplementation is very important at the end of the breeding season to reduce the incidence of EED.

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Systematic approach to efficiency problems in an embryo transfer program

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One of the main factors that affect efficiency is the scale of the embryo transfer (ET) program. The larger the program the more difficult becomes to keep all the variables that affect efficiency under control. In our program the efficiency improved constantly between 1997 and 2001. During these years the program did not grow very rapidly in terms of number of donors, so the general organization of the program could not only keep up with this growth but also became better organized each season. Management, facilities, nutrition conditions, labor skill improved steadily during this period. Recipient availability and quality also improved constantly until 2001.

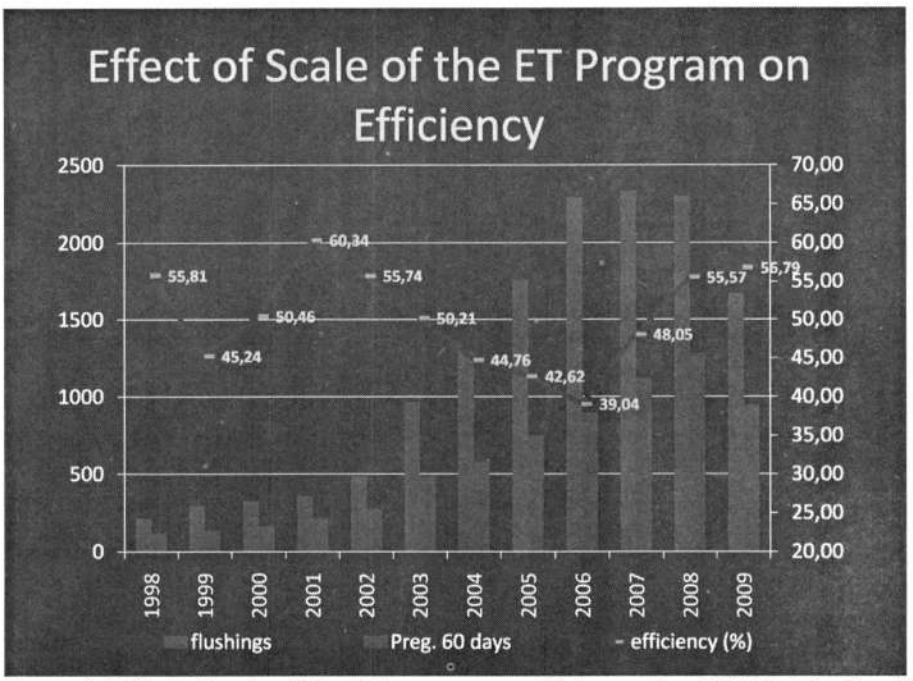

Figure 1. Effect of scale of the ET program on efficiency. (n= 13942). The line illustrates the efficiency. After 2001 the efficiency declined from 60% to39.04 % in 2006. After several changes efficiency climbed to reach 57% in 2009.

Between 2001 and 2002 our program started growing tremendously in terms of number of donors and obviously number of flushes performed per season (Figure1). This fast increase demanded changes in our facilities, management, labor, nutrition and many other aspects. These changes took time and were often delayed so the general efficiency of the program decreased. In order to keep up with the increasing demand we introduced drastic changes during 2007. These changes had an immediate impact on efficiency of the program. The most common parameters used to evaluate efficiency at Doña Pilar are embryo recovery, pregnancy at 14-21 days and embryonic and fetal loss (pregnancy loss) up to 60 days. Approximate figures for these parameters are: 1) embryo recovery = 70% for single ovulation cycles, 2) pregnancy (14-21 days)= 70-90% depending upon time of the year. 3) pregnancy loss = 8-15% depending upon time of the year.

Efficiency of an ET program can be expressed in terms of number of flushes averaged to produce a pregnancy in a certain period of time (flushes/pregnancy produced/time). Most of the

donors at Doña Pilar are enrolled in our program for a limited period of time to produce as many pregnancies as possible before returning to their athletic commitments or to be sold overseas.

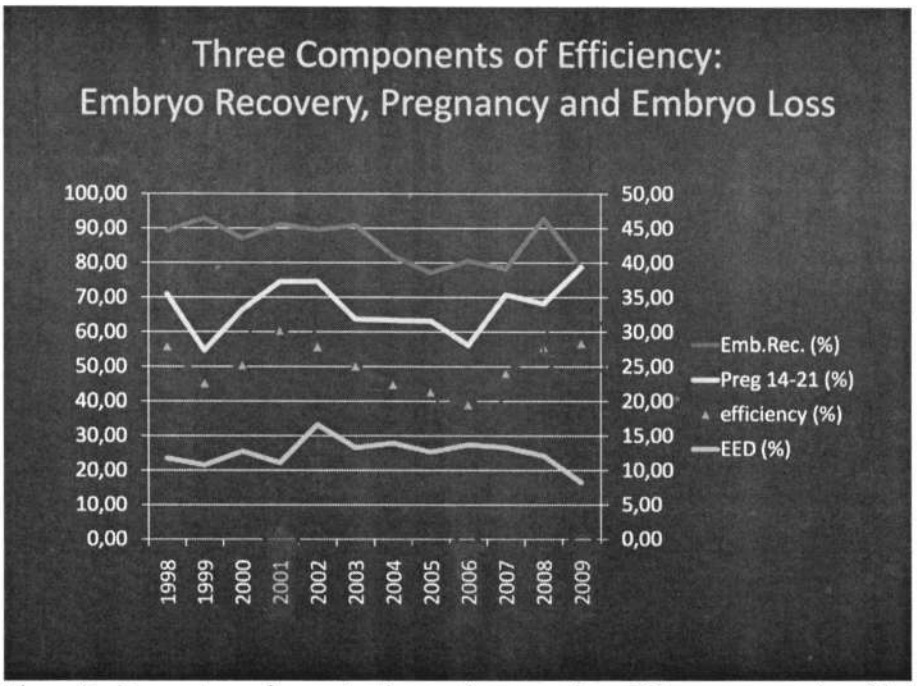

Figure 2. Parameters of reproductive performance in an ET program analyzed between 1998 and 2009. In the red line efficiency of the program is shown since 1998. Embryo recovery rate and pregnancy rate values are indicated on the primary y axis scale whereas EED rate is indicated on the secondary y axis. The analysis is concentrated on 2001, 2006 and 2009 because it better represents the effect of those factors that affect the efficiency.

Factors responsible for low efficiency in an ET program could be classified within one of the following five categories and can occur either individually or in combination and be transient or permanent throughout the season: 1) deficiencies in logistics and organization, 2) poor general horse management, 3) lack of sufficient quality recipients, 4) poor fertility of donors and stallions and 5) technical deficiencies.

The first three categories have the most impact on the results in a large scale program. It is not difficult to keep these categories under control when the program does not last too long and/or only involves a few horses. If the program lasts for the complete breeding season and involves hundreds of animals, it becomes a real challenge to keep the efficiency high without interruptions throughout the season. In contrast fertility of donors and stallions, technicians and procedures become very important if the program involves a small number of horses and the other aspects can be easily controlled. In new programs it is quite common to have several deficiencies combined and it usually takes some time and or expertise to detect which are the deficiencies and how to solve them.

Keywords: Embryo transfer, embryonic loss, commercial ET, reproductive management

Analysis of low embryo recovery rates

Normal rates of embryo recovery are approximately 70% per single ovulation cycle.[1] Careful analysis of breeding records would allow us to determine if the embryo recovery is low for all the mares or for only some individual mares. As it has been reported by many authors, embryo recovery rates can be influenced by many factors.[2-4] There are probably many others factors that are still to be

investigated. One of the questions that we wanted to investigate was if intensive ET during consecutive years could have a negative effect on reproductive performance of embryo donor mares.

Is there any negative effect of successive embryo transfer, seasons on reproductive performance of donor mares?

In a retrospective study involving 12,605 cycles in eight consecutive ET seasons, we studied the effect of intensive reproductive management of embryo donors on their fertility expressed as the ability to produce embryos recovered seven to eight days after ovulation and the pregnancy rate obtained with these embryos (Table 1). In addition, we studied the effect of successive ET seasons on embryo viability an incidence of pregnancy loss until day 60. The embryo recovery rate was not different in the group of mares during the first season of ET (82.84%) and mares that were embryo donors for eight consecutive seasons (81.25%). The pregnancy rate obtained from these embryos did not differ between groups. There appears to be no significant effect of consecutive ET seasons on embryo recovery and pregnancy rates at day 14. However, there appeared to be an increase in the incidence of pregnancy loss as the mares became older. In the group of mares enrolled as ET donors in their first year the early embryonic death (EED) was 12.59% whereas in the group of mares in their eighth consecutive year as embryo donors the EED was 22.39%.

Donor mares can be used during many successive ET seasons but as they become older it is sometimes more difficult to obtain embryos and pregnancies from some of them. In the author's experience, the most common problems are associated with the cervix and the uterus. That fact that these mares never foal has a negative influence on the cervical function and it can become hard and fibrotic in some cases and lacks the ability to relax and contract in response to progesterone levels throughout the estrous cycle. Another common problem in old mares is the increase in the susceptibility to endometritis. These mares are usually managed with minimal contamination techniques to prevent endometritis.[5]

Table 1: Effect of successive ET seasons on fertility of ET donors: (n=12,605 cycles)								
Season	Cycle	Recovered embryos	(%)	Pr 1	(%)	Pr 2	(%)	EED %
1	5827	4827	82.84	3146	65.18	2750	56.97	12.59
2	2782	2298	82.60	1527	66.45	1313	57.14	14.01
3	1600	1316	82.25	866	65.81	749	5691	13.51
4	1065	894	83.94	585	65.44	503	56.26	14.02
5	586	443	75.60	313	7065	257	58.01	17.89
6	359	279	77.72	190	6810	154	55.20	18.95
7	258	189	73.26	130	68.78	107	56.61	17.69
8	128	104	81.25	67	64.42	52	50.00	22.39
Total	12605	10350						

Is embryo recovery affected by multiple ovulation cycles?

Multiple cycles are considered very desirable in an ET program. We have observed over the years that although double ovulation cycles yield a higher embryo recovery rate it would not be double that of single ovulation cycles. To determine whether ovulation of two follicles from the same ovary (ipsilateral double ovulation) is associated with decreased embryo recovery in normally cycling mares, we retrospectively analyzed 1,300 double ovulation cycles.[6] We classified them as being ovulated from the same ovary (ipsilateral double ovulation) or from different ovaries (contralateral double ovulation). There was a tendency ($p=0.06$) for the incidence of ipsilateral double ovulation (699; 54%) to be higher than contralateral double ovulation (601; 46%). The embryo recovery rate

calculated per ovulated follicle was significantly higher in the bilateral than in the unilateral ovulation group (66% vs. 54%; p ,0.001). Embryo recovery was compromised if two ovulations occurred from the same ovary (ipsilateral double ovulation) and yielded a higher number of negative flushings compared to the contralateral double ovulation group (28% vs. 15%; p<0.001). Another conclusion of this study was that it appeared that there was a tendency for each breeding to produce either two embryos or not to produce any. This tendency was the same and independent whether the double ovulation was ipsilateral or contralateral.

In another study (Table 2) involving the analysis of 12,122 embryos obtained from single and multiple ovulation cycles, we analyzed the viability of embryos collected from single, double or triple ovulation cycles. We also included in the analysis two cases of spontaneous quadruple and quintuple ovulation. There were no differences in pregnancy rtes achieved and/or the incidence of EED in embryos obtained from single, double, triple, quadruple and quintuple ovulation cycles.

Table 2: Pregnancy rate and EED obtained from embryos collected from single and multiple ovulation cycles (n=12,122).

	Embryos	Pr 1 (n)	%	Pr 2 (n)	%	EED (%)
Single	8085	5486	67.85	4751	58.76	13.4
Double	3851	2624	68.14	2265	58.82	13.7
Triple	177	123	69.49	112	63.28	8.9
Quadruple	4	3	75.00	3	75.00	0
Quintuple	5	2	40.00	2	40.00	0

Are the donor mares fertile?

Depending upon the scale of the ET program we can assess if the embryo recovery rate is suboptimal for individual mares or for most mares. If we can determine that embryo recovery is low only for individual mares, then we need to examine these mares individually by performing a breeding soundness evaluation and reviewing the reproductive records if available. Although it is not the intent of this presentation to discuss all aspects related to mare infertility we have observed that the most common causes of mare infertility in our program are related to chronic endometritis and susceptibility to infections after artificial insemination. We treat most of these conditions with uterine lavages with physiologic saline or lactated Ringer's solution.[7] Oxytocin (20 IU iv) is given after each lavage.[8] Mares are inseminated using minimum contamination techniques as described.[5]

Ovarian disorders are rare. Anovulatory follicles can sometimes occur.[9,10] There are several known reasons for anovulatory follicles and probably many others that we do not know. In our practice most of the time anovulatory follicles are related to the use of anabolic steroids during competition or aged mares.

Oviductal function disorders are not very common or are rarely diagnosed.[11] We have seen mares with no evidence of reproductive disorder that consistently fail to produce embryos. We have attempted the endoscopic instillation of prostaglandin E_2 into the oviduct in several cases but we have not had any success as has been reported.[12] We have also detected donors that consistently produce embryos from one side and not the other suggesting the presence of a problem related to oocyte and embryonic transport through the oviduct. Cervical abnormalities are rare, with the most common being cervicitis related to many years of ET in old maiden mares. In these cases the cervix looses its competence to relax and subsequently fluid collects in the uterus after ovulation. We perform uterine lavages daily followed by iv injections of oxytocin. If fluid is still present by day five after detection of ovulation we perform uterine lavages on subsequent days incorporating an in-line embryo filter and we search for the embryo after the uterine lavage. Many mares with cervical lacerations can be suitable donors but they can be a real challenge to flush. When flushing these mares we keep our arm in the vagina and seal the uterus with the hand to prevent fluid loss into the vagina. If embryo recovery is low for most mares, and assuming that the entire group donor mares are not infertile, one should consider other causes that may not be necessarily related to mare fertility.

Are the mares cycling normally?

Mares are reliable ovulators once that the transitional phase is over. We have experienced cyclicity problems in mares being fed pastures with clover due to the effect of phytoestrogens. In these cases it is typical that follicles fail to ovulate and then regress. In severe cases we have seen mares discontinue ovarian activity which will resume if mares are changed to a different pasture. Aged mares may not cycle normally and fail to have follicular development. We have attempted hormonal supplementation with deslorelin acetate and progesterone but our results have not been very promising in these cases.

Our ET season usually starts at the end of September and extends until the end of April. If mares are exposed to 16 hours of light (natural + artifitial) they will start cycling in mid-August. The use of progesterone implants during the transitional phase has proven to be effective at promoting early ovulations. Anabolic steroids are sometimes used in polo pony mares during training and its long lasting effect on reproduction and ovarian activity is commonly seen at Doña Pilar.

Quality of reproductive management

The next step on the analysis of low embryo recovery rates is the evaluation of the reproductive management. We can do this by evaluating the following parameters in the breeding records.

When are donor mares being inseminated in relation to ovulation? Mares are inseminated within 48 hours before ovulation is detected particularly if semen of stallions of normal sperm longevity is being used. Post-ovulation insemination sometimes occurs when mares ovulate unexpectedly. In these cases embryo recovery is usually lower if the mare has not been examined every six hours. In a limited study performed at Doña Pilar, we evaluated the effect of post-ovulation breeding on embryo recovery.[13] Mares were examined by trans-rectal palpation and ultrasonography of the internal genitalia twice daily 12 hours apart. Mares that ovulated unexpectedly were immediately inseminated, so the maximum interval between ovulation and insemination was 12 hours. Post-ovulation breeding yielded a significantly lower embryo recovery rate (63 vs. 83%). There was no significant difference in the proportion of grade 1 embryos between the groups (76/85 [89%] pre- ovulation vs. 13/19 [68%] post-ovulation. The use of ovulating inducing agents such as human chorionic gonadotropin and deslorelin aid in the management of insemination so very few mares ovulate unexpectedly. Although post-ovulation breeding yields lower embryo recovery it is a better option than skipping the cycle. If mares are to be inseminated with frozen semen or semen from stallions with short sperm longevity we intend to inseminate as close to ovulation as possible.

When are uterine flushes performed in relation to the detection of ovulation? Flushes are scheduled to be performed seven to eight days after detection of ovulation.[14] Embryos recovered are usually at the stage of early to expanded blastocysts and the size ranges between approximately 200 and 2000 microns. If the flush is performed too early (day six or less) the embryo recovery will be lower. The reasons for a lower embryo recovery rate at day six are: failure of the embryo to descend into the uterus, failure to recover the embryo due to a higher specific weight or failure to recognize the embryo.

Table 3. Effect of flush date on reproductive parameters (n=16,195)

Flush date	Number of flushes	Number of positive flushes	Positive flushes (%)	Number of embryos recovered	Embryo recovery (%)	Preg at 14-21 days	Preg (%)	Preg at 60 days	EED (%)
6	277	169	61.01	203	73.29	132	65.02	108	18.18
7	5850	3800	64.96	4525	77.35	3196	70.63	2786	12.83
8	8807	5331	60.53	6490	73.69	4302	66.29	3760	12.60
9	1111	548	49.32	654	58.87	444	67.89	371	16.44
10	150	65	43.33	72	48.00	51	70.83	39	23.53

In some situations flushes are performed either before day seven or after day eight:

Post-ovulation breeding. Mares that have been inseminated after ovulation are usually flushed one day later (day eight or nine after detection of ovulation).

Severe endometritis. Mares with severe endometritis that collect intreuterine fluid after ovulation and do not respond to regular therapy are usually flushed earlier to reduce the time the embryo spends in the uterine environment. In many cases the uterus is lavaged daily after ovulation and oxytocin treatment is instituted. Embryos usually descend into the uterus approximately 155 hours after ovulation,[15] so uterine lavages performed from day five onwards include an in-line embryo filter in order to search for the embryo in case it is collected.

Embryo vitrification. Embryos can be successfully vitrified at the stage of late morulas to early blastocysts.[16] Embryo vitrification by conventional methods yields very poor results once the embryo size exceeds approximately 240 microns In order to recover embryos suitable for vitrification uterine flushes should be performed at day six after ovulation.

Multiple ovulations. The flush is usually scheduled so the embryos will be between 6.5 to 8.5 days. We have collected older embryos (day nine and ten), but special considerations are necessary to maintain viability of these embryos. If multiple ovulations have occurred more than three days apart it is probably better to do two different flushes.

Aged mares. Development of embryos is frequently delayed in aged mares[14] so if a mare repeatedly fails to produce an embryo when flushed at day eight or 8.5 we will flush them on day nine or ten. Embryos from these mares appear to be delayed in development and yield a lower pregnancy rate and a higher EED rate (Table 3).

Artificial insemination with frozen semen. Mares inseminated with frozen semen are usually flushed at Day 8.5 or nine after detection of ovulation. This is because embryos appear to develop at a slower rate when are produced by insemination with frozen semen.

Stallion fertility

If the ET program involves few stallions their intrinsic fertility will have a profound effect on the overall results of the program. The effect of an individual stallion is diluted if the program involves many different stallions. Thus, the first step for analysis in cases of low embryo recovery rates regarding stallions is to evaluate them individually. A complete breeding soundness evaluation including analysis of existing records is essential. At Doña Pilar we charge clients on a per pregnancy basis, so we suggest our clients avoid using subfertile stallions unless we can get reasonable results by instituting different treatments and management approaches. The most common problems related to stallion fertility we have seen at our clinic are: low libido, low sperm numbers, urine contamination and decreased longevity of sperm motility.

Even with stallions of proven fertility there are still differences in embryo recovery rates. In a retrospective study involving 14 of the most popular stallions in our program that have been involved in 250 flushes or more, the rate of positive flushes differs among stallions and ranges between 63% and 78%.

If the embryo recovery rate is low for all stallions then the problem is probably not related to the stallions' fertility and the investigation should proceed.

Table 4: Reproductive performance of stallions involved in an ET program (n=7400 flushes)

	Total Flushes	Positive Flushes	%	ET	PR1	(%)	PR2	EED(%)
Bagual	641	449	70.05	531	365	68.74	318	12.88
Compinche	317	201	63.35	232	148	63.79	126	14.86
Durazno	451	284	62.97	362	209	57.73	186	11.00
Zorrino	668	485	72.60	595	369	62.02	311	15.72
Gold Macy	319	219	68.65	249	195	78.31	167	14.36
Granado	325	254	78.12	317	207	65.30	179	13.53
Tintero	523	351	67.11	407	310	76.17	252	18.71
Triple Equis	693	439	63.32	501	383	76.45	329	14.10
Xenon	548	394	71.87	458	344	75.11	285	17.15
Perugino	257	198	77.04	240	181	75.42	161	11.05
River Slaney	1492	984	65.94	1160	784	67.59	693	11.61
Signo	275	213	77.41	269	164	60.97	149	9.15
Solcito	367	232	63.22	269	168	62.45	153	8.93
Theol	1168	805	68.92	973	666	68.45	592	11.11

Semen collection. All materials used for semen collection including collection bottles, semen filters, sterile lubricant and artificial vagina (AV) liners should be non-spermicidal. We have experienced problems with some types of lubricants. We prefer to use disposable plastic bags (Whirl Pak®, Nasco, Fort Atkinson, WI) instead of collection bottles. At Doña Pilar we use primarily the Missouri model artificial vagina (Nasco) but other models are sometimes used. The collection procedure is very important not only because it can be dangerous for both the operator and the stallion if it is not performed properly but also because stallions may develop behavior and fertility problems produced by deficient management in the breeding shed during semen collection. Many times low embryo recovery rates associated with a particular stallion is not related to the stallion's intrinsic fertility but to how the stallion and the semen have been collected and processed.

Semen processing and evaluation. All materials in contact with semen must be non-spermicidal. We have used commercial extenders or we have made our own following Kenney's formulation. It is important to assure that each ingredient is proven to be non-spermicidal. We have experienced problems with different batches of antibiotics and brands of skim milk. Cell culture grade water is recommended to prepare semen extender.

Flushing technique. If donor mares and stallions have been ruled out as the causes for a low embryo recovery rate, and management of artificial insemination and semen processing have been also investigated, the analysis should concentrate on the flushing technique.

Uterine flushing is not a difficult procedure for an experienced veterinarian. It is important to use the proper equipment. Integrity of the inline filters, particularly if they are recycled should be checked, since broken filters will result in loosing embryos during collection. In our practice inline filters are recycled many times. Filters are evaluated routinely under the dissecting microscope to find defective filters which are immediately discarded.

No less than 95% of the infused medium should be recovered during flushing. It is better to develop a regular routine and continue flushing without interruption while the operator creates a slight

turbulence in the uterus by gently moving his/her fingers. Failure to obtain a continuous flush may be due to over-inflation of the cuff. Moving the catheter slightly forward by pushing on the back of the cuff can help in finding fluid pockets within the uterus. It can be difficult to completely recover the infused volume in some mares. The author usually manually compresses the left horn to move the fluid towards the right horn followed by pushing the catheter forward into the right horn by pressing the caudal part of the cuff using the thumb while the rest of the hand keeps the left horn and the body of the uterus compressed. Then all the remaining fluid is in the right horn and the tip of the catheter is pushed forward into this horn. When the tip of the catheter reaches the tip of the right horn the uterine flush can be completed. This maneuver can only be performed by using very flexible catheters that will not irritate the cervix or the uterus. Some situations can result in difficult flushes such as:

1) Full bladder: it is important to evaluate the mare before the flush while the rectum is cleaned of feces. If the bladder is filled with urine it will make the procedure much easier if the bladder is emptied first by means of a catheter.

2) Open or lacerated cervix: if the cervix is not intact it may not be able to hold the fluid in the uterus during the flush. In this case we perform the uterine flush without taking the arm out of the vagina. We grasp the cervical os with a finger around the catheter preventing the fluid from being lost into the vagina.

3) Difficult recovery of infused medium is common in older mares or mares flushed on the first cycle after foaling. If the mare has a large pendulous uterus it may be difficult to collect all the infused medium. In these cases we prefer to use another type of catheter. The cuff should not be inflated with air since this will keep the catheter floating on the top of the fluid against the uterine wall. It is better to inflate the cuff with medium so the catheter will be submerged in the medium facilitating collection.

Analysis of low pregnancy rates after transfer

It is very common when dealing with efficiency problems in an ET program, to collect embryos at normal rates, but fail to produce pregnancies after transfer. In such cases the investigation should focus systematically on several aspects of the program.

Are horses being properly managed?

It is a common mistake when analyzing poor results to underestimate the importance of proper general horse management in an ET program. Deficiencies In general management will induce stress that will lower efficiency and it is one of the major factors affecting the efficiency of a large scale program. The most common effects of stress due to deficient horse management in an ET program are decreased pregnancy rate after transfer, reduced embryo viability after transfer and an increase in EED. Many mares will go into anestrous sooner at the end of the season if general management conditions are suboptimal. Control of stress and maintenance of good horse management have been major challenges in a large ET program. Housing conditions, water and shade availability, sanitation, nutrition and feeding and general management are essential to obtain good results in an embryo transfer program.

Nutritional status of the recipient herd

Pregnancy rates can be dramatically affected in recipient mares that are losing weight, even when they are in good body condition. Nutrition level also affects EED rates, which can be high if the mares are losing weight.

Repeated transfers to the same recipient

If the ET program is experiencing low pregnancy rates, recipients that are diagnosed not pregnant after transfer are immediately reused in the next cycle. In our experience, providing that the recipients have passed a breeding soundness evaluation and have not shown any evidence of abnormality, they can be transferred again. Assuming that the transfers were performed properly using sterile materials and a clean technique, non-surgical transfer is not a harmful procedure and does not affect immediate fertility if the mare did not become pregnant after the transfer. Pregnancy rates after transfer among recipient mares that received one, two, or three embryos during the same season were not significantly different.

Are materials and media proven to be non-toxic?

All material and tubes should be sterile and free of residues toxic to embryos. Although many materials are disposable, equipment that is to be reused is washed with de-ionized water and sterilized. Tubes, catheters, and filters are sterilized with ethylene oxide for 24 hours. Ethylene oxide residues are extremely toxic to sperm, embryos and humans so it is critical that materials are ventilated for no less than two weeks before being reused. We experienced serious problems due to ethylene oxide toxicity using materials that had not been properly ventilated.

The embryo should not be exposed to direct sunlight. Ultraviolet light can damage embryos and sperm and compromise their viability. Temperature control is also mandatory since temperature shock can damage both spermatozoa and embryos. Laboratories are kept at approximately 24°C. Holding medium, stored in a refrigerator is warmed in an incubator to 35°C as is any other material in contact with the embryos. Once the flush is completed we take the medium out of the incubator so it cools to room temperature while the embryo is being located and processed.

Transfer technique

There is a significant effect of technician skill on pregnancy rates achieved by non-surgical ET.[18] An experienced technician could achieve a 75% or higher pregnancy rate when every factor affecting pregnancy rate is under control. This is not a difficult figure to achieve during a limited period. However optimal conditions are quite difficult to maintain during the entire ET season in a large scale program, so overall pregnancy rates at the end of the season are usually around 65-70%.

In a retrospective study involving 12,122 embryos the pregnancy rate was not very different between transfers classified as quality 1 (perfect transfer) and 2 (only slight difficulties) (68.3 vs 67.2%). However if the transfer difficulty was classified as grade 3, the pregnancy rate was significantly lower (41.07 %).

Table 5. Effect of transfer skill on pregnancy rates and EED (n=12,122 embryos)

Transfer Skill	Total	PR 1	(%)	PR 2	(%)	EED (%)
1	11281	7701	68.27	6668	59.11	13.41
2	418	281	67.22	243	58.13	13.52
3	56	23	41.07	20	35.71	13.04

Embryo quality

Because the incidence of abnormal embryos is low compared with that in other domestic species where superovulation treatments are used,[19] the effect of morphologic abnormalities on the general results should not be significant. Still embryo quality has to be considered in individual cases when attempting to find a reason for failure of a recipient to become pregnant after embryo transfer.

In a retrospective study involving 11,810 embryos classified morphologically in three categories (grade 1, 2 and 3) the pregnancy rate was higher for grade 1 embryos (68.88%) than for grade 2 (59.3%) or grade 3 embryos (3224% [Table 6]). The incidence of EED was also different among these three categories. Since embryos with severe morphological abnormalities can still produce pregnancies, we do not discard embryos based on morphological features. All embryos are transferred despite their quality since our center is concerned about the number of pregnancies not pregnancy rates.

Table 6. Effect of embryo quality on pregnancy rates and EED.
(n=11810)

	Total	Pr 1	%	Pr 2	EED (%)
Grade 1	10,884	7497	68.88	6527	12.94
Grade 2	774	459	59.30	362	21.13
Grade 3	152	49	32.24	37	24.49

Oval embryos. Sixteen embryos were classified as oval embryos with no other morphological feature. The pregnancy rate achieved with these embryos was 75% (12/16). It is not clear which is the reason for this "abnormality" although it may be related to osmotic changes in the medium.

Embryo age. As it was previously discussed, embryos can be successfully collected from the uterus between day six and eight after detection of ovulation and in some selected cases even at day nine and ten. Embryos grow very fast at this stage. In a retrospective analysis of 10,884 grade 1 embryos we classified them in eight categories depending upon the size and the stage of development (Table 7). There was no difference on pregnancy rates and EED rates achieved within these categories with the exemption that the embryos classified as morulaes appeared to have the highest EED rate.

Table 7. Effect of embryo size on pregnancy rate and EED after transfer of
Grade 1 embryos (n=10,884)

	Total	Pr 1	(%)	Pr 2	EED (%)
m1	172	94	54.65	75	20.21
eb1	1086	745	68.60	608	18.39
< 550 μ	3332	2412	72.39	2091	13.31
550–1000 μ	3911	2718	69.50	2381	12.40
1000–1500 μ	1849	1176	63.60	1059	9.95
1500–2200 μ	382	252	65.97	230	8.73
2200–2900 μ	90	61	67.78	51	16.39
>2900 μ	62	39	62.90	32	17.95

Morulas. Morulas are not commonly found when flushing mares from day seven onwards. Even in mares flushed at day six the incidence is low although higher than flushings performed on following days (Table 8). Only 254 morulas were found out of 11810 embryos collected (2.1%). Pregnancy rates achieved with these embryos are usually lower if there are compared with pregnancy rates obtained with early blastocysts and expanded blastocysts (Table 9). The incidence of morphological abnormalities is higher in morulas (33%) than in the other categories of embryos

collected. The incidence of EED is also higher (20.21%, 33.33% and 40.0% in morulas classified as grade 1, 2 and 3, respectively). The higher incidence of embryonic abnormalities and EED in morulas collected at day seven (Table 9) onwards is probably due to the fact that normal embryos should be at an advanced stage of development, which is an indication that at least some of these embryos are in the process of EED.

Table 8. Proportion of different grades of embryo quality depending on embryo size (n=11,556)

	Total	Grade 1	(%)	Grade 2	(%)	Grade 3	(%)
m	254	172	67.72	59	23.23	23	13.37
eb	1382	1086	78.58	229	16.57	67	6.17
< 550 µ	3690	3332	90.30	326	8.83	32	0.96
550–1000µ	4016	3911	97.39	96	2.39	9	0.23
1000–1500 µ	1908	1849	96.91	42	2.20	17	0.92
1500–2200 µ	393	382	97.20	9	2.29	2	0.52
2200-2900 µ	97	90	92.78	5	5.15	2	2.22
>2900 µ	70	62	88.57	8	11.43		

Table 9. Reproductive parameters with morulas depending on flush date (n=244)

Flush day	Morulas	Preg (14-21 days)	Preg (%)	Preg at 60 days	EED (%)
6	19	12	63.16%	9	25.00%
7	119	63	52.94%	51	19.05%
8	91	28	30.77%	18	35.71%
9	13	5	38.46%	4	20.00%
10	2	2	100.00%	2	0.00%

If morulas have a lower pregnancy rate after transfer, it is not surprising that pregnancy rates achieved with vitrified embryos are also lower compared with those obtained with fresh embryos. When analyzing efficiency in vitrification programs it is important to acknowledge that pregnancy rates are lower with fresh morulas. This is just another indication that EED is an ongoing process that starts after fertilization. Flushing the uterus allows observation of what is going on at day seven to nine. Ultrasonography permits investigation of EED from day ten onwards. Embryonic death at the time of fertilization and flushing is more difficult to evaluate. How many of the negative flushes correspond to lack of fertilization? How many embryonic deaths occur between fertilization and flushing? How often do we fail to collect the embryo from the uterus, or fail to find it in the uterine lavage? These questions are still to be answered and the factors involved remain unknown.

Seasonality

The first consideration is that in the Southern Hemisphere the ET season starts in mid-September and ends in mid-May. The busiest time of the season is between December and March. When analyzing the efficiency it is important to consider whether it has been uniform throughout the season or if there have been times during the season when the efficiency was lower than normal.

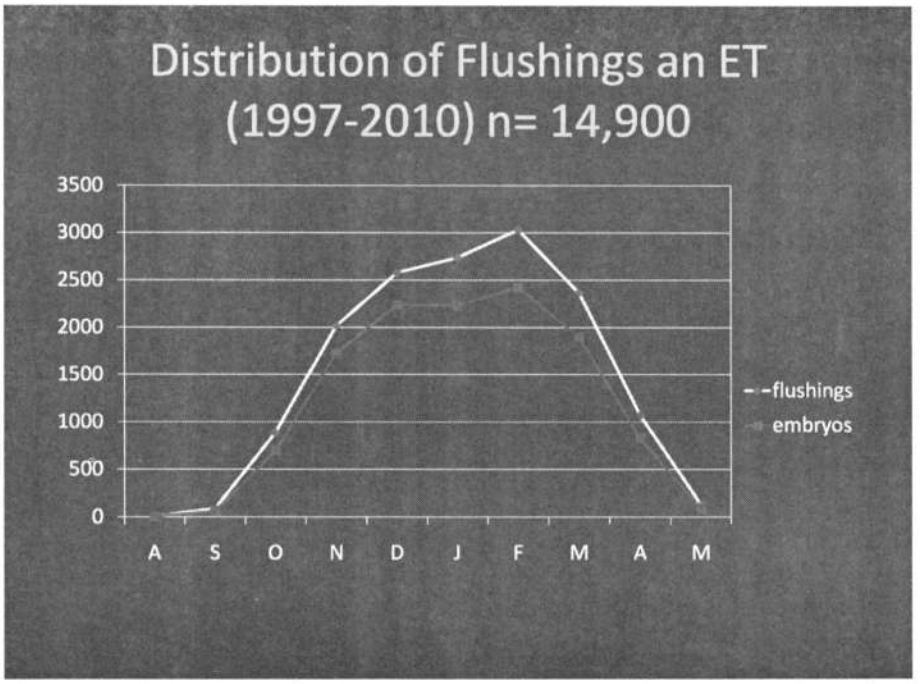

Figure 3. Distribution of flushes and ET during the breeding season. The peak of the ET season is between December and March. In the blue line are the number of flushes and in the red is the number of embryos recovered

If the embryo recovery rates throughout the season from the start to finish are considered it appears that the embryo recovery rate remains quite stable. If two seasons (2006 and 2009) are compared both lines follow the same trend and are not very different form the average obtained from 1997 to 2009.

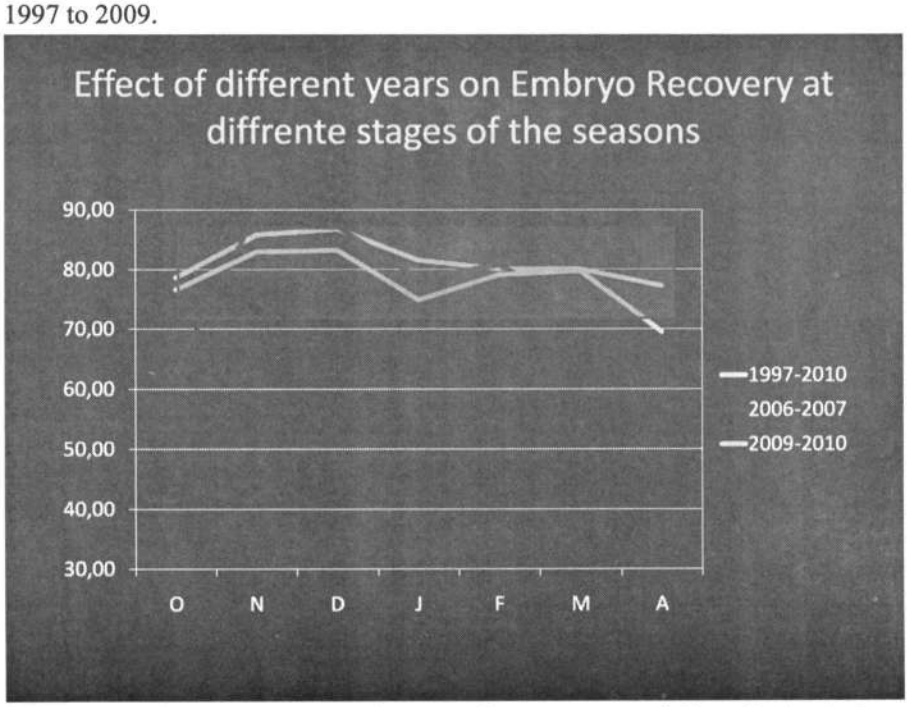

Figure 4. Embryo recovery rate does not change very much from the beginning to the end of the season even when comparing years of good and poor efficiency.

However when pregnancy rates after transfer were compared, a difference among ET seasons was noted. The pregnancy rates are higher during the spring and the difference between 2006 and 2009 occurs between December and the end of the ET season in April. The pregnancy rate in 2006 decreased rapidly while in 2009 the pregnancy rate was maintained close to 70%. The question is why? What did we change after 2006 to improve the efficiency in 2009?

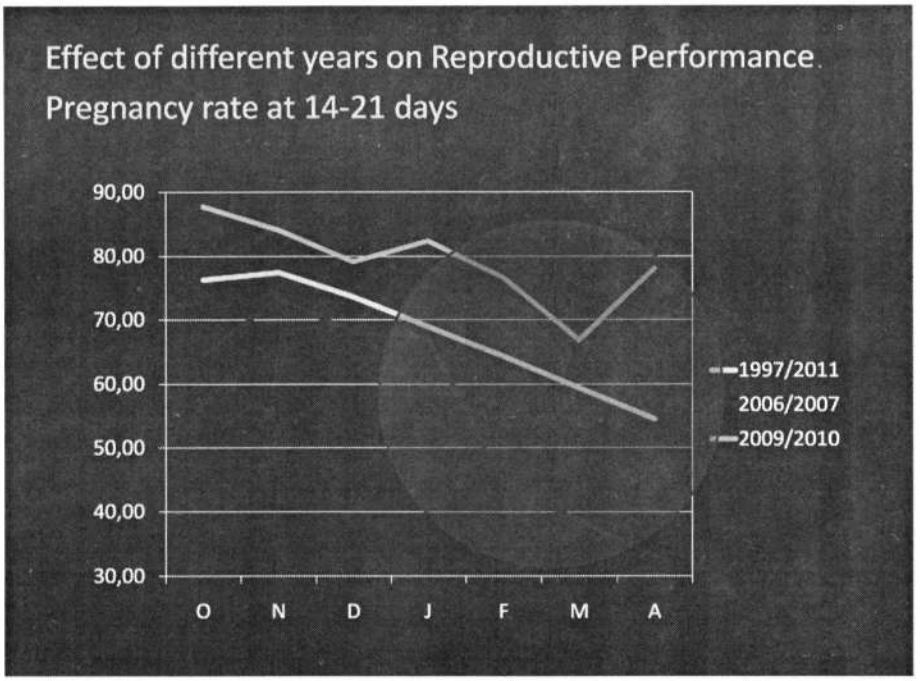

Figure 5. The difference between good and poor ET seasons has been due primarily to differences in pregnancy rates and EED rates from December to the end of the season.

There are several known reasons why pregnancy rates are higher during the spring than in the summer and fall. One of them is related to the quality of the recipients. During the spring there are more recipients available. Since the best recipients are usually used first the recipient pool becomes smaller as the ET season progresses. This situation can be controlled in advance if the program has enough good recipients that can be evenly distributed along the season.

Heat stress is an important factor that affects embryo viability after transfer. As has been shown in other species (cattle for example), heat stress has a negative effect on fertility. This effect can be overcome with good general management, providing shade and water and selecting the best hours (early in the morning or late in the afternoon) to perform most of the activities with horses.

Social stress is also an important factor in our program. Many recipient mares are added to the program during the summer after weaning their previous ET foal. Some of these mares have poor body condition scores when admitted to the program.

Sanitary conditions are very important. Although mares are routinely vaccinated against strangles, new mares added to the program are sometimes affected by this disease which will compromise their ability to become and maintain pregnancy.

During the early fall some mares will cycle erratically or will enter anestrous. We use progesterone supplementation on almost every recipient at this stage. Long acting progesterone (1.8 g per week) is given after ET. Pregnant mares are kept on progesterone until day110 when progestagens produced by the placenta can maintain the pregnancy. In some cases progesterone supplementation is discontinued after secondary corpora lutea have been identified.

Table 10: Effect of time of the year on reproductive parameters in an ET program. (n=14900)

	Flushes	Embryos	(%)	Pr 1	(%)	Pr 2	(%)	EED (%)
August	6	5	83.33	4	80.00	4	80.00	0.00
September	88	61	69.32	38	62.30	32	52.46	15.79
October	884	695	78.62	530	76.26	468	67.34	11.70
November	2014	1729	85.85	1339	77.44	1247	72.12	6.87
December	2578	2239	86.85	1647	73.56	1484	66.28	9.90
January	2736	2232	81.58	1538	68.91	1334	59.77	13.26
February	3031	2425	80.01	1561	64.37	1288	53.11	17.49
March	2366	1893	80.01	1124	59.38	894	47.23	20.46
April	1067	824	77.23	449	54.49	380	46.12	15.37
May	130	84	64.62	44	52.38	33	39.29	25.00

Analysis of early embryonic death

Since embryonic death is a process that starts immediately after fertilization and occurs throughout the embryonic stage it is not surprising that the same factors that affect pregnancy rates after transfer will also affect embryonic death rates.[20] Most of these factors have been discussed above. In some instances factors affecting EED rates are related to the intrinsic fertility of the animals involved (donor, stallion and recipient). In these cases particularly in donors and stallions, there are few options for intervention. But there are other cases when EED rate is due to technical and management factors. In our experience managerial changes have produced a profound effect on the incidence on EED and pregnancy loss before day 60 which is the time when pregnant recipient are usually discharged from our program and are sent home for foaling.

Stallion factors

As it has been noted above we intend to work with fertile stallions in our program but even with a fertile population it is possible to detect differences among stallions in pregnancy rates and EED. Intensive reproductive management will help some stallions with short sperm longevity achieve good embryo recovery rates. However we have encountered other stallions with low pregnancy rates after transfer and high incidence of EED.

Donor mare factors

We have detected donor mares that produce embryos at normal rates but it appears that these embryos have lower viability and experience a very low pregnancy rate after transfer and a high incidence of EED. As mentioned above we detected an increase in the incidence of pregnancy loss as mares become older. For those mares enrolled as donors in their first year EED was 12.59% whereas in the group of mares in their eighth consecutive year as embryo donors the EED was 22.39%. (Table 1).

In a retrospective analysis of our records we studied the group of donors with highest incidence of EED. We included in the analysis only donors that had been flushed on at least 20 occasions and that had experienced an incidence of EED greater than 20%. The percentage of positive flushes ranged from 47.77 to 100%. Most of these mares were old and had a high incidence of multiple ovulations. The pregnancy rate ranged between 57.89 and 81.25% (Table 11).

Table 11. Reproductive parameters in mares selected for high EED rate

Donor	Flushes (n)	Positive flush (%)	Embryos recovered (n)	Embryos recovered (%)	Preg 14-21 days (n)	Preg 14-21 days (%)	Preg 60 days (n)	EED (%)
Alegria	47	66.04	35	74.47	23	65.71	18	21.74
Araña (BC)	35	79.55	35	100.00	24	68.57	18	25.00
Capota	44	71.74	33	75.00	24	72.73	18	25.00
Felicidad	63	47.77	32	50.79	23	71.88	18	21.74
Gama	49	64.82	35	71.43	22	62.86	17	22.73
Imperial	35	78.05	32	91.43	26	81.25	19	26.92
Malapata	71	61.85	47	66.20	28	59.57	21	25.00
Maty	86	69.24	72	83.72	50	69.44	39	22.00
Moneda	20	100	28	140.00	21	75.00	16	23.81
Patito	24	90.7	29	120.83	21	72.41	15	28.57
Power Mina	36	73.81	31	86.11	23	74.19	17	26.09
Rose	27	100	44	162.96	33	75.00	26	21.21
Sara	52	79.04	49	94.23	38	77.55	30	21.05
Surra	34	88.24	45	132.35	28	62.22	22	21.43
Vertiente	47	69.1	38	80.85	22	57.89	17	22.73

Embryonic factors

In a study involving embryos graded according morphological features we found no difference in EED between embryos grade 1 and 2. However there was a high incidence of EED in grade 3 embryos. In a retrospective study involving 11,810 embryos classified morphologically in three categories (grade 1, 2 and 3) the pregnancy rate was higher in grade 1 (68.88%) than in grade 2 (59.3%) or grade 3 (32.24%). The incidence of EED was also different among these three categories. There was no difference in pregnancy rates and EED rates achieved within these categories with the exemption of the embryos classified as morulas.

Seasonality

As has been previously noted, embryonic death has a seasonal pattern. Even during very good ET seasons EED increases in the summer and fall at end of the ET season (Table 12, Figure 6). Progesterone supplementation appears to be beneficial at controlling EED to some extent. Heat stress, as has been shown in cattle and other species can dramatically affect EED rates. The importance of good horse management to prevent or decrease the decline in embryo survival that occurs during the summer and fall cannot be overemphasized.

Table 12: Effect of month of the year on EED rate (1997-2010).

	1997-2010 (%)	2006-2007 (%)	2007-2008 (%)	2008-2009 (%)	2009-2010 (%)
October	11.70	7.69	10.00	13.21	11.63
November	6.87	5.70	7.24	6.87	3.66
December	9.90	13.70	7.14	8.83	7.11
January	13.26	18.04	5.88	14.52	6.47
February	17.49	23.72	18.26	10.97	11.50
March	20.46	14.17	31.43	20.92	7.08
April	15.37	8.16	16.09	18.18	12.50
May	25.00	0.00	27.27	26.67	37.50

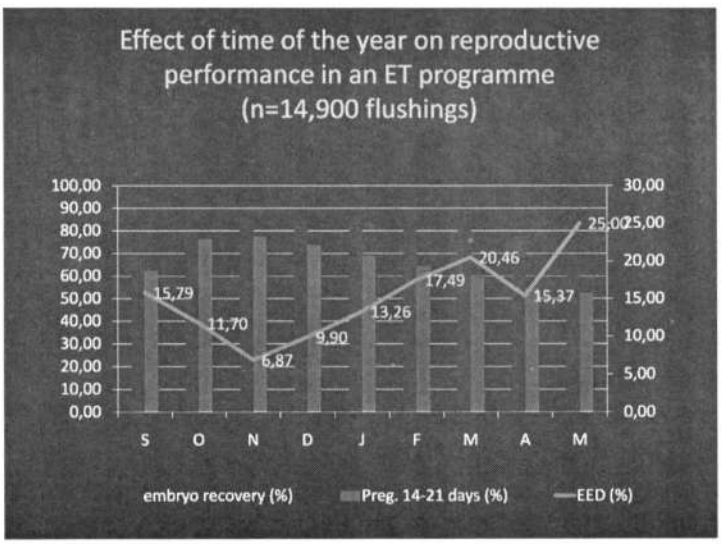

Figure 6. Effect of season on reproductive performance.

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Late gestation pregnancy loss in the mare

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Abstract

Efforts should be made to determine the etiology of any abortion. The aborting mare should be isolated until the cause of the abortion is determined to not put other pregnant mares at risk. Examination of the aborting mare will direct appropriate therapy and prepare the mare for future breeding. The entire abortus should never be frozen but kept chilled if timely submission to a diagnostic laboratory is possible. Thorough abortus examination and proper sample submission will help make the diagnosis. Management and medical prevention of equine abortion is discussed.

Keywords: Abortion, equine, necropsy, twins, herpesvirus, placentitis

Introduction

Abortion in late gestation represents an economic and management loss to the breeder. Once an abortion is recognized an immediate effort must be made to identify the etiology and determine if there is risk to other pregnant mares on the farm. Knowledge of the cause of the pregnancy loss should also direct the mare's therapy and management to prevent future pregnancy loss. Reviews of the causes of pregnancy loss in mares are available,[1,2] however this paper will direct the clinician on how to manage the aborting mare, obtain a diagnosis and develop a management plan to prevent abortions.

Pregnancy loss detection

Traditionally breeding farms had strong mare teasing programs that were used throughout the breeding season. Not only did these teasing programs provide information about when a mare should be bred, sexually receptive behavior observed in the pregnant mare band may have been the first sign of pregnancy loss. Outside of the breeding season detection of a loss would be obvious if a mare had fetal membranes hanging from the vulva or an aborted fetus or membranes in their immediate environment. Otherwise the loss may only be detected when the mare was found to not be pregnant at a periodic pregnancy examination. Many but not all aborting mares have premature mammary gland development prior to or at the time of fetal expulsion. In some cases mammary development may be the only sign of impending abortion and may direct the veterinarian to closely monitor the pregnancy. It is prudent that all abortions be investigated to rule out the presence of contagious disease that may cause loss in other pregnant mares. Steps should be taken to manage the aborting mare, the abortus and other pregnant mares on the farm.

Mare management

The aborting mare should be kept isolated from other pregnant mares until the etiology of the abortion is determined to not be contagious. Strict hygiene and biosecurity measures should be followed to prevent the spread of possible infection to other horses. The aborting mare should have a physical examination to identify any systemic illness or infectious disease. Clinical signs of systemic disease (i.e., fever, respiratory aberrations, depressed appetite) may no longer be evident by the time the fetus is expelled. A blood sample should be obtained in an evacuated serum tube (Vacutainer® serum tube, Becton, Dickinson and Company, Franklin Lakes, NJ) at the time of the abortion. A second serum sample may need to be obtained in two to three weeks if a diagnosis is not made and paired serum samples are necessary for additional diagnostic tests. The genital tract should be examined by palpation and ultrasonography per rectum and palpation per vagina to detect the character of the uterus, any retained fetal membranes, retained fetus (twin, mummy), uterine fluid/exudate and trauma to the cervix and pelvic canal. Unless aerobic culture of an endometrial swab is performed in the immediate post-abortion period (<24 to 48 hours) the results of the culture are unlikely to reveal the cause of the abortion. Although routine histological evaluation of an endometrial biopsy sample at this stage may reveal

inflammation it usually does not reveal the cause of the abortion and therefore is not indicated. If fetal membranes are retained low doses of oxytocin (10-20 IU) can be repeatedly administered repeatedly intravenously or intramuscularly. If retention has been longer than eight hours, broad spectrum antibiotics and flunixin meglumine (1.1 mg/kg iv SID) should be administered until after membranes have passed. If there is partial retention of fetal membranes, the uterus should be lavaged daily with several liters of sterile saline to help dilute and decrease the amount of uterine exudate and dislodge the retained piece of fetal membrane. Mares that have uterine fluid would also benefit from uterine lavage, administration of ecbolics and exercise to help evacuate the uterus.

If the mare aborted in a stall the environment that was contacted by the abortus should be cleaned with disinfectants, dried and the bedding burned or disposed appropriately. Virus in the environment is unlikely to survive in an infectious form after 21 days.[3] If the abortion took place in a field, an attempt should be made to cordon off the affected area from other pregnant mares. The mares in that field should not be moved to another location until the incubation period of any infectious disease has passed without additional clinical problems and equine herpesvirus (EHV), equine viral arteritis (EVA) and leptospirosis have been ruled out as the cause of the pregnancy loss. The American Association of Equine Practitioners has guidelines for Infectious Disease Outbreak Control to help prepare your management plan.[4]

Diagnostic evaluation

A gross examination of the fetal membranes should be made.[5] Gloves should be worn and the examination should be made in a site that can be disinfected. Note if the fetus is inside the amnion and allantois. The chorion should be have microvilli over its entire surface except for the avillous areas at the cervical star, opposite the insertion of the umbilical cord, at the oviduct papilla sites and any folded areas. In a normal term delivery the end of the gravid horn typically is edematous and the microvilli may appear sparse. A large bare area on the chorion devoid of microvilli may indicate a twin placenta that should prompt one to look for a second fetus and second set of fetal membranes. Scrutinize the cervical star area and note marked edema and abrupt lines of demarcation separating areas of different color and character that may indicate an ascending placentitis. Note any discrete areas of exudate especially thick, brown, mucoid exudate between the base of the horns or at what had been the most ventrally located portion of the membranes which might indicate a focal mucoid placentitis. Invert the membranes to examine the allantois. Measure the umbilical cord noting the length of the amniotic and allantoic portions. The normal range of umbilical cord length in a full term Thoroughbred mare is 36–83 cm.[5] The umbilical cord always has some twists but excessive twisting with evidence of thrombus formation or constriction may indicate umbilical cord torsion. Note the character of the amnion. Is there evidence of meconium staining that may indicate fetal distress in utero? If possible the fetal membranes should be weighed. Normally fetal membrane weight at term is approximately 11% of the foal's body weight. Edema or placentitis can increase the weight of the fetal membranes.

The whole abortus should be kept cool (not frozen) and submitted to a diagnostic laboratory as soon as possible. Provide a serum sample from the mare that can be paired with a convalescent serum sample should additional testing be required to identify an infectious etiological agent. History should be provided to the laboratory diagnosticians that includes the stage of gestation, general health and vaccination history of the mare, exposure to sick horses, previous pregnancy loss or infertility problems. The pathologists should be informed if other pregnant mares are at risk for exposure to infectious disease agents. Proper contact information should be provided so both preliminary and final results can be communicated to the individuals responsible for managing the pregnant mares.

If the entire abortus cannot be submitted to a diagnostic laboratory in a timely fashion, a field necropsy should be performed to harvest the tissues needed to optimize obtaining a diagnosis of the etiology of the abortion. Many diagnostic laboratories will provide abortion submission kits to practitioners in remote locations to have available to facilitate a field necropsy and direct the submission of appropriate tissue samples to optimize the chance of obtaining an accurate diagnosis. The diagnostic laboratory should be contacted prior to submission to inquire if polymerase chain reaction (PCR) and

immunohistochemistry tests are available and if samples require special handling. Although laboratories may have different methods of sample submission the following directives can be a general guideline.

After gross examination of the fetal membranes a 2 cm^2 sample should be taken from the cervical star, body, gravid horn and nongravid horn of the chorioallantois, the amnion and umbilical cord and placed in formalin for histological evaluation. A sample of chorioallantois should be placed into virus transport medium (VTM) for EHV and equine arteritis virus isolation. A sample of chorioallantois should be submitted frozen if the laboratory can perform PCR for EHV and leptospirosis. Equine arteritis virus PCR requires that the tissue be fresh.

The fetus should be grossly examined, weighed and the crown-rump length measured. Presence of hair should be noted to help estimate the stage of gestation. Presence of any meconium should be noted. Place the fetus in right lateral recumbency and reflect the left forelimb and hind limb. Incise the body wall behind the ribs reflecting as much of the abdominal body wall and thoracic wall without touching the underlying viscera. Aspirate any peritoneal, pleural or pericardial fluid and place in a labeled red top Vacutainer® tube. Aseptically collect a 2 cm^2 piece of spleen, liver, lung, and thymus for fluorescent antibody testing and PCR and place each sample in a separately labeled plastic bag (Whirl-Pak®, Nasco Fort Atkinson, WI) containing VTM. For leptospira, PCR samples of vitreous humor and kidney (cortex and medulla) are placed in transport medium. Collect a pooled set of all the above tissues and place into VTM for virus isolation. Aseptically place a large piece of lung in a plastic bag for aerobic bacterial culture but do not add VTM to this sample. Snip a hole in the stomach and using a transport culture swab obtain gastric fluid for culture.

After inspection of each organ, place a 2 cm^2 piece of kidney, spleen, heart, thymus, adrenal, stomach, eyelid, tongue, skeletal muscle, small and large intestine and brain and several larger sections of lung and liver into 10% formalin for histological evaluation. Retain a large piece of liver and vitreous humor for later possible toxicological analysis. All of the samples harvested should then be packed in an insulated container on frozen freezer packs and transported by overnight courier to the diagnostic laboratory. Laboratories may be able to screen rapidly for EHV using PCR, immunohistochemistry staining and histology.

Abortion prevention

Viral abortion

Not all pregnancy losses can be prevented but there are general management steps that can be taken to decrease the incidence of pregnancy loss. The causes of infectious viral abortion in mares include EVH-1, equine arteritis virus, EVH-4 and equine infectious anemia virus. To decrease the chance of exposure to disease one should limit the addition of new mares to the pregnant broodmare herd. New animals should be quarantined for at least three weeks before turn out with any broodmare band. Pregnant mares should be subdivided into physically separated small groups for the duration of gestation. Mare bands should be kept separate from transient horses. In particular pregnant mares should be isolated from young stock in training and performance/competition horses. It is recommended that pregnant mares be administered a killed vaccine against EVH-1 at 5, 7 and 9 months of gestation.[6] Some farms that have endemic problems with EVH-1 abortions, farms with large numbers of pregnant mares or a transient herd population may elect to vaccinate against EHV every other month to prevent clinical disease. Studies have shown that a modified live EHV vaccine is safe to administer to pregnant mares but its use is not approved to prevent abortion in pregnant mares.[7] Unfortunately abortions due to EVH-1 still occur in mares vaccinated against EHV-1 but vaccination does seem to prevent large abortion outbreaks.

Although vaccination against equine arteritis virus can prevent EVA abortion, routine administration of EVA vaccines is not recommended for all broodmares.[6] Equine arteritis infection is spread by aerosolized nasal droplets or infected semen. Mares exposed to the virus will be viremic for up to 40 days and if pregnant may abort. After recovery from the initial infection there seems to be no effect on future fertility and mares do not become chronic virus shedders. Infected stallions will seroconvert

and shed the virus in the semen for a few weeks. Some EVA positive stallions become chronic shedders as the virus seems to persist in the secondary sex glands and shedding seems to be testosterone dependent. Stallion managers should determine the EVA status of their stallions and provide that information to all mare owners planning to breed mares to their stallion. If the EVA titer is negative, the official document stating that negative status should be retained as part of that horse's permanent record. The stallion should then be vaccinated (four weeks before breeding) and annual boosters administered no earlier than four weeks before the start of the breeding season. First time vaccinated horses need to be isolated from direct contact with nonvaccinated horses for three weeks. It is strongly recommended that seronegative mares be vaccinated at least three weeks prior to being bred to a seropositive equine arteritis virus shedding stallion. The manufacturer does not recommend the use of EVA vaccine in pregnant mares especially in the last two months of gestation. Seronegative pregnant mares should be prevented from coming into contact with equine arteritis virus shedding horses. Any horse that may be considered for export should have an EVA titer performed to determine its EVA status prior to EVA vaccination. The official titer report and vaccination record should be maintained in that horse's permanent record.

Regular surveillance for equine infectious anemia should be performed by requiring that all contact animals have a negative Coggins test.

Bacterial abortion

Bacterial infections that end in pregnancy loss are usually due to some form of placentitis. Bacterial placentitis tends to occur in three forms, diffuse, focal and ascending placentitis. Systemic bacterial infection with a bacteremia can cause inflammation throughout the uterus. This results in a diffuse inflammation of the chorion and may progress to a funisitis (inflammation of the umbilical cord) and amnionitis. *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Streptococccus spp.*, *Staphylococcus spp.*, *Salmonella abortis equi* and *Leptospira spp.* can cause diffuse placentitis.[2] Prevention of diffuse placentitis depends on prevention of the initial bacterial infection.

Clinical signs of illness are not usually apparent before abortion due to leptospirosis. Infected cattle, swine and wild animals such as opossums, deer, raccoons and skunks shed leptospires in the urine and contaminate water sources. A pregnant mare infected with leptospires becomes bacteremic and develops a diffuse placentitis resulting in abortion. A mare that aborted should be isolated as the abortus contains leptospires and the mare may shed the organisms in urine for many weeks. Exposed pregnant mares found to have elevated titers should be administered oxytetracycline (5 mg/kg) intravenously once a day or procaine penicillin G (20,000 IU/kg) intramuscularly twice a day for seven to ten days.[2] Mares should be provided clean water and prevented from drinking at contaminated water sources.

Nocardioform or focal mucoid placentitis is seen as a focal area of necrosis of the chorionic microvilli covered by a brown, opaque, thick, mucoid exudate located at the most dependent aspect of the gravid uterus at the junction of the uterine horns. A nocardioform actinomyte, *Crossiella equi* and other similar organisms have been associated with the focal lesion. Mares may have premature mammary development but otherwise may not show other outward clinical signs. Areas of increased placental thickness and exudate can be seen ultrasonographically. The pathogenesis of focal mucoid placentitis is not understood and methods to prevent the initial infection are not known.

Ascending placentitis results from a bacterial or fungal infection that enters the caudal genital tract and infects the caudal uterine body portion of the placenta. The pregnancy outcome depends on the stage of gestation and how much of the conceptus is affected. If the infection is extensive the fetus may become infected and abortion occurs. If only a portion of the chorioallantois is affected the clinical problem is one of placental insufficiency. In many cases the infection is localized to the cervical star and caudal uterine body resulting in a delay in the rupture of the chorioallantois at the time of parturition or even premature separation of the chorioallantois (red bag delivery). Mares may have premature mammary gland development and vulvar discharge. Prevention of ascending placentitis is based on maintaining competency of the caudal genital tract. The mare's perineal conformation must be carefully evaluated at the time of breeding. If the vulvar seal is incompetent an episioplasty should be performed to prevent air from aspirating into the vagina. In most cases a Caslick's procedure may be adequate but

some mares may require a Gadd procedure that will augment the perineal body. Some mares' perineal conformation may appear fine in the summer when the mare is being maintained on good grass pasture and is in good body condition. But if the mare loses body condition after summer and fall pastures wane one should evaluate the quality of feedstuffs, and the mare's dental condition, parasite load and general comfort. Necessary changes should be made to keep the mare in good physical and body condition. Mares at risk for ascending placentitis should be monitored daily for evidence of vulvar discharge and premature mammary gland development. The uterine body and cervical star region of the placenta should be evaluated repeatedly by ultrasonography per rectum for evidence of an increase in thickness of the uteroplacental unit and accumulations of exudate at the cervix. Administration of appropriate antibiotics should be administered if evidence of placentitis is present. Other steps to manage placentitis should be considered. Mares that develop urovagina in late gestation when the abdomen sags and pelvic canal relaxes will likely develop an ascending placentitis. These mares should have a urethral extension prior to rebreeding or very early in the next pregnancy. The owner/manager of a mare that has had perineal surgery should be reminded that an episiotomy may need to be performed before parturition. This is usually done approximately two weeks before the mare's due date or when the mammary gland starts to develop. Mares with very poor perineal conformation may need to have a few large simple interrupted sutures (that can be easily removed at parturition) placed in the labia to keep them apposed after the episiotomy.

Twin abortion

Although some twin pregnancies may result in the birth of two live foals most twin pregnancies result in abortion or neonatal loss. Late term abortion of twins may put the pregnant mare at risk for dystocia. Regardless of there being some breed, age and management predilection for twinning, all mares should be examined carefully for twins. Even if only one ovulation was detected at the time of breeding, mares bred to a stallion with good fertility may asynchronously ovulate a second follicle days later resulting in a second conceptus. The use of ultrasonography for early pregnancy evaluation has decreased the incidence of pregnancy loss due to twinning but unfortunately the problem does still exist. The American Veterinary Medical Association Professional Liability Insurance Trust (AVMA PLIT) has recommended the following to help increase the chance of detecting twin pregnancies.[8] For best results, mares should be scanned twice to minimize the risk of missing twins, once at 17-23 days after breeding and again at 28-30 days. If economics dictate a single examination, it should be performed between 25-30 days of gestation. Owners should be always warned that even for the most experienced ultrasonographers it is not always possible to be 100% certain that twins are not present. Performing an ultrasound examination twice reduces the risk considerably but cannot guarantee to totally eliminate the chance of twin pregnancy. AVMA PLIT also recommended that an accurate measurement of the embryonic vesicle be made and ascertain that the embryo is the appropriate size for the stage of pregnancy. A thorough understanding of the ultrasonographic character of the developing equine conceptus will help the examiner recognize the presence of twins.[9]

Effort should be made to optimize the ultrasound examination. The mare should be comfortably restrained in a dimly lit area. The ultrasound settings should be adjusted to achieve the best resolution of the image. The ultrasound unit should be positioned at eye level in front of the examiner. It is important that one carefully examines the entire uterus consciously starting at the tip of one horn next to its ipsilateral ovary, tracing that horn to the bifurcation and then tracing up the entire length of the second horn to the tip ipsilateral to the second ovary. Care must be taken to examine the uterine body immediately in front of the cervix. In mares with a pendulous uterus one may want to position the ultrasound probe ventral to the uterine horn to be sure any sacculations at the base of the horn are thoroughly examined.

Practical reviews of twin pregnancy management are available.[10] If twins are detected prior to 30 days of gestation, as high as 90% of the manual twin reductions can result in a singleton at term.[11,12] Between 25 and 53 days of gestation transvaginal aspiration of one twin may result in as high as 55% of the twins being present shortly after aspiration but the foaling rates of a singleton may be much lower at

term.[13] Cervical dislocation of one twin performed between 55 and 90 days of gestation per rectum or through a flank incision at 58 to 150 days resulted in a singleton at term in 63% of the cases.[10,14] After 90 days of gestation intracardiac injection of KCl or procaine penicillin into the smaller or more poorly positioned twin via transabdominal needle guided ultrasonography may allow 40 to 50 % of the remaining twins to develop to term.[15] These later procedures provide options for twin pregnancies that are missed in the first month but the best results are obtained if one twin is manually reduced prior to 30 days of gestation.

Summary

It is prudent to determine the etiology of all abortions so one can rule out the presence of infectious disease and develop a management plan to prevent future abortions.

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Brain masculinization is independent of genital masculinization in the fetal sheep

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In sheep, prenatal exposure to testosterone masculinizes both the external genitalia and the ovine sexually dimorphic nucleus (oSDN) of the hypothalamus-preoptic area of the fetal brain. The present study tested the hypothesis that temporally separate critical periods exist for masculinization of the external genitalia and the brain. Pregnant ewes were treated weekly with 200 mg testosterone propionate (TP) either from Day 30 to 60 (Early TP) or Day 60 to 90 (Late TP) of gestation. As a control (C), pregnant ewes were treated with oil during the same gestational periods. At gestational day 135 ± 2.0 (SEM), the fetuses were delivered by Cesarean section. The oSDN was identified by its characteristic expression of aromatase mRNA using in situ hybridization and by thionin staining. Grossly, Early TP female fetuses possessed a penis and a scrotum devoid of testes, whereas Late TP and C female fetuses had phenotypically normal external genitalia. Neither period of exposure to TP grossly affected the genitalia of male fetuses. Despite the masculinized genitalia, the volume of the oSDN in Early TP females (0.32 ± 0.06 mm^3) did not differ from C females (0.24 ± 0.02 mm^3), but was significantly enlarged in the Late TP females (0.49 ± 0.04 mm^3) even though their genitalia appeared normal. In contrast, the oSDN from Late TP males (0.51 ± 0.02 mm^3) was not different from C males (0.51 ± 0.04 mm3), but was significantly smaller in the Early TP males (0.35 ± 0.04 mm^3). These results demonstrate that the prenatal critical period for sexual differentiation of the oSDN occurs later than, and can be temporally separated from, the critical period for masculinization of the external genitalia. This information expands our understanding of prenatal brain (behavioral) and phenotypic sexual differentiation. A disruption of endocrine signals during fetal critical periods, whether naturally occurring or iatrogenic, may result in an animal that appears normal yet displays sexual behavior that is not typical of their phenotypic gender.

Keywords: Ovine sexually dimorphic nucleus, sheep, sexual differentiation, hypothalamus, external genitalia

Effect of deslorelin implants on domestic queen puberty: a preliminary report

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Prolonged administration of GnRH agonists acts through desensitization and down-regulation of the GnRH pituitary receptors. However, this procedure is initially preceded by an increased release of gonadotrophins which, in females, can result in an estrous response. Long-term release GnRH agonists have shown to postpone puberty in boys,[1] although their effect on feline puberty has not been assessed. We hypothesized that the long term release GnRH agonist, deslorelin acetate, postpones puberty in queens without the initial stimulation of the gonadal axis, when administered when the cat is at approximately 50% adult body weight. The aim of this study was to assess the efficacy and safety of deslorelin acetate implants on domestic queen puberty postponement.

Thirty 90 to 180 day old, 1.3 to 1.6 kg prepubertal crossbred female cats were included in this study during a year and one-half period. Five of the cats (17%) were littermates. The animals were kept under a positive photoperiod (14L:10D) since birth, and after weaning they were fed a commercial kitten food and given water *ad libitum*. The study was approved by the Faculty Institutional Care and Animal Use Committee.

The females were randomly assigned to one of the following groups: deslorelin acetate 5 mg SC implants (Ovuplant®, PepTech Animal Health, Macquarie Park, NSW, Australia; n=15) or to a non-treated control group (n=15).

The queens were followed up daily and weighed weekly until puberty. Vaginal cytology was also carried out three times a week or whenever estrous signs appeared. Puberty was diagnosed by the presence of the typical estrous behavior in the presence of a tom cat and vaginal cytology. Age (days) and weight (kg) at puberty (mean±SEM) were compared between groups by Student's t-test. The level of significance was set at 0.05.

Age but not weight at puberty differed between deslorelin-treated and control groups, respectively (237.8±14.1 vs. 177.8±10.8; $P<0.01$ and 2.6±0.1 vs. 2.56±0.1; $P>0.1$). Two deslorelin-treated queens developed pyometra 13 and 92 d after implantation, respectively. The remaining animals did not have any side effects.

At the time of writing, four deslorelin-treated, 251±20 days old, cats have not reached their puberty yet; these animals already differ from control queens ($P< 0.01$). Another female of the same group did not achieve puberty after implantation 18 months ago, this cat was considered an outlier and excluded from the previous analyses.

It was concluded that these deslorelin implants administered at this particular body weight range seemed to postpone ($\geq$ 6 weeks) feline puberty without altering growth. Although side effects were low (<15%), they should be considered when using these implants.

Key words: Cat, deslorelin, feline, GnRH agonist, puberty

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Effects of a 200 µg dose canine gonadotropin releasing hormone vaccination on mares

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Introduction

Equity™ (Pfizer Australia Pty, Ltd, West Ryde, NSW, Australia)) is an equine gonadotropin releasing hormone (GnRH) vaccine (containing 200 µg of GnRH peptide) labeled for estrus suppression available in Australia and New Zealand.[1] Canine Gonadotropin Releasing Factor Immunotherapeutic™ (Pfizer Animal Health, Exton, PA) is a canine GnRH vaccine labeled for benign prostatic hyperplasia manufactured in the U.S. Our laboratory has previously shown that a 5X dose of the canine GnRH vaccine (1000 µg of GnRH peptide) safely suppresses estrous cyclicity in mares.[2] The objective of this study was to determine if a lower dose of the canine GnRH vaccine (200 µg of GnRH peptide; the labeled canine dose) would suppress estrous cyclicity in mares. Our hypothesis was that GnRH antibody titers would be lower but still sufficient to prevent equine estrous cyclicity and behavior.

Materials and methods:

During early Spring 2010, mares with a history of extreme estrous behavior (n=18) received two (1 ml) intramuscular injections in the neck of Canine Gonadotropin Releasing Factor Immunotherapeutic™ vaccine at a 30 day interval. Jugular venous blood samples were collected at time 0, 30, 90, and 150 days. Serum progesterone was measured using the chemilluminesence assay and GnRH antibody titers were determined by an enzyme linked immunosorbent assay previously validated in our laboratory. At day 90, transrectal ultrasound examinations were performed to determine ovarian structures and a questionnaire was given to owners to determine change in behavior.

Results

There were no adverse reactions to vaccination in any of the mares. At day 90, 17/18 mares (94%) were in anestrus (defined as no follicles measuring >20 mm and no luteal tissue visible using transrectal ultrasonography and a serum progesterone concentration of <0.5 ng/ml). Sixteen owners (89%) responded that the mares' estrous behaviors were greatly reduced since initial vaccination. During the middle of summer (at day 150), progesterone concentrations remained <0.5 ng/ml in these mares, suggesting that they were still not cycling. Forty-four percent of mares had GnRH antibody titers ≥1:16.

Conclusion

Administration of 200 µg of GnRH peptide in the canine GnRH vaccine is an effective way of immunologically preventing estrous cyclicity in most mares, even those with very low (undetectable) antibody titers. This method is especially useful for performance athletes as well as mares that express extreme estrous behavior.

Keywords: Estrous behavior, GnRH, mare, progesterone, vaccination

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Determination of canine placental blood flow using pulsed wave Doppler ultrasonography

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Introduction

Ultrasonography is an important non-invasive tool used to monitor the fetal health during canine pregnancy.[1] However, reports on gestational changes in umbilical artery blood flow during normal pregnancies are contradictory.[2,3] In addition, blood flow within the canine placenta has not been reported. The aim of this study was to describe changes in placental, umbilical, and uterine artery blood flow during the last third of gestation in normal canine pregnancies. Our hypothesis was that blood flow would increase in all of these vascular beds with increasing gestation.

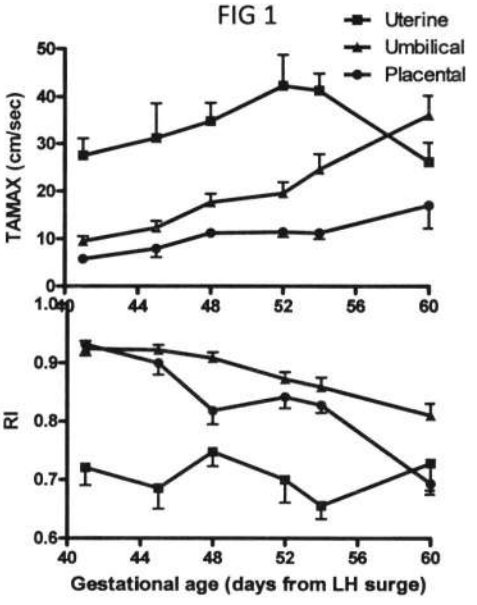
Materials and methods

Time-averaged maximum velocity (TAMAX) and resistance index (RI) were determined from placental, umbilical and uterine arteries from pregnant beagles (n=4) using pulsed wave Doppler ultrasonography (Mindray M5, Shenzhen Mindray Bio-Medical Electronics C., LTD., Nanshan, Shenzhen, China) with a 5-8 MHz micro-convex transducer twice weekly from 41-60 days past the LH surge (term=65 days). Data were compared using a one-way ANOVA (GraphPad Prism®, GraphPad Software, Inc., La Jolla, CA) and $P<0.05$ was considered significant.

Results

Uterine blood flow (TAMAX) and RI did not significantly change during late gestation (Figure). However, both umbilical and placental blood flow increased, although umbilical blood flow was greater than placental blood flow. In addition, vascular resistance within both the umbilical and placental arteries decreased with gestation, with lower resistance in placental arteries compared to umbilical.

FIG 1



Conclusion

The results from this study are similar to previous research that concluded that umbilical TAMAX increases during gestation.[4] However, other investigators had found that uterine artery vascular resistance decreases while uterine artery blood flow increases with gestational age,[2,4] which differs from our results. It is important to note that this is the first report to describe changes in blood flow through canine placental arteries during late gestation. These data may be useful for clinicians following high risk pregnancies as it has been shown in humans that placental vascular resistance is higher in cases of placental insufficiency.[5]

Keywords: Canine, Doppler ultrasound, placental artery, umbilical artery, uterine artery

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The role of strain-specific adhesin genes in binding of pyometra-inducing *E. coli* to canine endometrium

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Escherichia coli (*E. coli*) is the most commonly isolated infectious agent causing pyometra in bitches. The adhesin *FimH* has been shown to facilitate bacterial attachment to canine endometrium.[1] However, many *E. coli* strains isolated from the uteri of infected dogs carry several adhesin genes (*fimH*, *papGIII* and *sfa*). The objective of this study was to investigate the role of each adhesin gene product, acting alone or expressed in combination, in the bacterial binding to canine endometrium.

E. coli strain P3, which was isolated from a uterus of a bitch naturally affected with pyometra, was shown by PCR to carry all three known adhesin genes. Knockout (KO) mutants of this wildtype (P3-wt) strain were generated using insertional inactivation. Single (P3-Δ*fimH::Kan*; P3-Δ*pap::Cm*; P3-Δ*sfa::Kan*), double (P3-Δ*fimH::Kan*-Δ*pap::Cm*; P3-Δ*fimH::Kan*-Δ*sfa::Kan*; P3-Δ*pap::Cm*-Δ*sfa::Kan*), and triple (P3-Δ*fimH::Kan*-Δ*pap::Cm*-Δ*sfa::Kan*) mutants were produced. Adhesion assays on anoestrous uteri of three post-pubertal bitches were undertaken. Full-thickness tissue samples were collected using a 6 mm biopsy punch. Tissue samples from each uterus were washed separately in PBS and incubated with the P3-wt or P3-KO strains, or with PBS as a negative control. After washing, tissue samples were homogenized and plated on nutrient agar for determination of colony forming units (CFU)/cm^2 of tissue.

Overall, the number of bacteria adhering to canine endometrial biopsies were comparable and no significant difference in the number of bound bacteria was found between the P3-wt strain and the single or double KO-strains. However, the triple knockout strain (P3-Δ*fimH::Kan*-Δ*pap::Cm*-Δ*sfa::Kan*) displayed less binding to the canine endometrium compared with the P3-wt strain ($p=0.034$; by Dunnett's simultaneous test).

This study shows that a pathogenic *E. coli* strain (P3) isolated from the uterus of a bitch with pyometra was able to fully compensate for the loss of two of its three known adhesin genes. It was necessary to inactivate all three known adhesin genes in order to see a significant decrease in binding to canine endometrium. However, the triple knockout mutant still retained 42% binding capacity compared with the P3-wt strain.

This retained binding contrasts with similar analyses of a different *E. coli* strain, P4, in which functional loss of the single adhesin gene (*fimH*) eliminated >99% of bacterial binding capability.[1] These combined studies suggest that pathogenicity varies between *E. coli* strains. Therefore, future research should focus on the interaction of bacterial virulence and host immunity in order to further elucidate the pathogenesis of canine pyometra.

Keywords: Adhesin, dog, *E. coli*, pyometra

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Safety and efficacy of a controlled release deslorelin acetate product (SucroMate™) for induction of ovulation in mares

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Deslorelin acetate (da) implants (Ovuplant) were shown to release this GnRH analog (da) for an extended period of time resulting in suppressed concentrations of FSH and reduced follicular activity and extended interovulatory interval in some mares after Ovuplant treatment. This study evaluated the safety of administering 1x (1.0 mL), 3x (3.0 mL) and 5x (5.0 mL) dose of SucroMate™ Equine (CreoSalus, Inc., Louisville, KY). SucroMate™ Equine is composed of 1.8 mg da in 1.0 mL sucrose acetate isobutyrate (SAIB):propylene carbonate (70:30 Wt:Wt). Thirty-two mares were assigned to one of four doses (placebo, 1x, 3x, 5x da) and received the same dose IM for three consecutive estrous cycles once mares had an ovarian follicle of 30 to 40 mm and had been in estrus at least two days. Variables measured to assess animal safety included complete physical examinations, heart and respiratory rate and rhythm, body temperature, nine general observations associated with potential adverse reactions, hematology and clinical chemistry, and urinalyses. Skin temperature and reaction at injection site were taken prior to each treatment and one, three and six h after treatment and daily for seven days until day 21. There were no differences between da dose groups and the placebo group with respect to any of the variables measured to assess animal safety during any of the three treatment cycles. All injection site reactions, which were mild to moderate for all groups, had resolved for mares in the 1X group by three days following treatment. Blood samples were collected for measurement of LH and FSH prior to treatment, six to 12 hr after treatment, twice-daily during estrus, and every third day after ovulation until the next estrus, or day 18 of pregnancy. Data were analyzed by repeated measures analysis of variance. The profiles of LH in the SucroMate™ Equine treated groups in all three cycles exhibited an expected surge following treatment and a gradual return to pretreatment levels by 24 hr. For days three to 18 post-ovulation, the values of LH in the da groups were similar to those in the placebo group. At 6-12 hr post-injection mares in the 1X group had higher ($p<0.05$) FSH compared to all other groups. During all three treatment cycles, concentrations of FSH during days three to 18 after treatment were similar for 1X and placebo groups. Time to ovulation and duration of estrus was shortened in all da treated mares compared to placebo mares (85 and 21% ovulated within 48 hr). The interovulatory interval in da mares ranged between 18 to 23 days compared to 21 to 24 days for those treated with placebos. Number of mares pregnant was 6 of 7 (86%), 6 of 6 (100%), 6 of 8 (75%), and 5 of 5 (100%) for placebo, 1X, 3X and 5X da groups, respectively. In conclusion, administration 1.8 mg deslorelin acetate (1X) was safe and effective for induction of ovulation during three consecutive estrous cycles with no adverse effect on animal safety, decrease in FSH during diestrus, change in interovulatory intervals, or decrease in pregnancy rates.

Keywords: Deslorelin, ovulation, mares

Three-day progesterone-priming protocol for synchronization and superovulation of goats during transition

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Synchronization of estrous cycles and superovulation are important tools for the reproductive management of goat herds. Many traditional synchronization-superovulation protocols call for extended periods of progesterone priming that may last anywhere from nine to 21 days. We hypothesized that dramatically shorter periods of progesterone priming would produce similar or better results than traditional longer priming. The objective of this study was to compare superovulation results using a short (three day) versus a long (11 day) progesterone priming protocol. Parameters measured included numbers of unovulated follicles, ovulation points, and embryos, and unfertilized ova. Blood was drawn daily to determine serum progesterone levels.

Ten female goats were divided into two groups receiving controlled internal delivery drug releasers (Eazi-Breed CIDR Sheep & Goat; Pharmacia and Upjohn, New York, NY), for either three days (short protocol) or 11 days (long protocol). One control animal in each group received the same treatments as the four experimental does, except for the CIDR. All does were superovulated with 1.6 mL follicle stimulating hormone (FSH; Follitropin-V; Bioniche, Belleville, ON) im bid for four days, starting two days before the removal of the CIDR and continuing for one day after. Because the experiment was conducted in June (transition), the does were not expected to be cycling, but 5 mg $PGF_2\alpha$ (Lutalyse; Pfizer, New York, NY) was given im on the day of CIDR removal in order to assure that no corpora lutea were present. Fifty μg GnRH (Cystorellin; Merial, Duluth, GA) was given im the day after the last FSH administration to induce ovulation. All does were naturally bred by the same buck at the first sign of estrus and every 12 hours thereafter until they were no longer receptive. Surgical collection of embryos via caudal laparotomy was performed two days after administration of GnRH. Embryos were collected using a retrograde flush of the oviduct with 20 ml of embryo collection medium (Biolife 'Advantage,' AgTech, Manhatten, KS) and evaluated. One experimental animal was dropped from each group due to CIDR loss, and surgical complications.

There were no significant differences between the short and long progesterone groups for any parameter. The data means and standard deviations are shown below:

Protocol	Follicles	Ovulation points	Embryos	Unfertilized ova
Long (n=3)	7±5.3	10.7±5.5	9±8.5	1.3±-2.3
Short (n=3)	9±6.0	17.7±6.0	11.3±5.9	0.3±0.6

Neither control animal produced embryos. At the start of FSH administration progesterone levels were higher in the short protocol group than in the long protocol group.

The results of this study suggest that a three-day progesterone priming protocol is a viable method for synchronizing and superovulating goats during transition. Further research is needed to investigate potential differences between the long and short protocols, including possible relationships to progesterone levels, and embryo viability.

Keywords: Goat, CIDR, estrus synchronization, progesterone, superovulation

Parturition increases serum amyloid A concentration in healthy pregnant mares

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Serum amyloid A (SAA) is a major acute phase protein and its concentrations are usually below 7 mg/L in healthy horses. However, SAA concentration can increase quickly and with larger amplitude in response to inflammation than other acute phase proteins (e.g. fibrinogen). Therefore, SAA has the potential to be a sensitive indicator of presence and magnitude of inflammatory/infectious disease during pregnancy in mares. Our hypothesis was that that normal parturition would induce a rapid rise in circulating SAA in healthy pregnant mares. Objectives of the study were: 1) to determine baseline values for SAA in pregnant mares during the last eight weeks of gestation, and 2) to determine if normal parturition affects SAA concentration.

Fifteen healthy Warmblood mares between nine and 17 years of age were used in this study. Mares were inseminated with fresh or frozen semen and ovulation was detected by ultrasonography. Blood was collected weekly starting on Day 280 of gestation until parturition, and then at 12, 36 and 60 h postpartum. Blood samples were collected from the jugular vein, centrifuged at 800 *g* for 10 min, and the serum was collected and stored at -80°C. At the time of blood collection, mares were submitted to a physical evaluation (i.e. determination of rectal temperature, capillary refill time and respiratory and heart rates) to ascertain overall health. In addition, mares were evaluated by ultrasonography per rectum every other week to determine fetal viability and combined thickness of uterus and placenta (CTUP). Three measures of the CTUP were performed by an experienced practitioner and the average measurement was recorded. Serum concentrations of SAA were determined using a commercial ELISA kit (Tri-Delta Diagnostics Inc., Boonton Township, NJ) according to the manufacturer's instructions for equine serum. Data were normalized for parturition date and the information obtained during the last eight weeks of gestation and 60 h post-partum were used. Data were compared by paired t-test and results expressed as mean ± sem. Significance was set at $P<0.05$.

During the period of observation, the CTUP significantly increased from 8.4 ± 0.6 mm (8 weeks pre-partum) to 9.7 ± 0.2 mm (2 weeks pre-partum), but values were within normal limits for this stage of gestation. As expected, the average concentration of SAA remained at baseline (i.e. <7 mg/L) during the last 8 weeks of gestation. There was a significant increase in serum concentration of SAA within 12 hours of parturition (62 ± 26 mg/L), and concentration remained elevated at 36 h post-partum (188 ± 111 mg/L). However, concentration of SAA returned to basal levels within 60 h postpartum (7 ± 4 mg/L).

In conclusion, SAA remains at baseline during late pregnancy in the mare. In addition, normal parturition induces a significant rise in SAA concentration within 36 h post-partum, but SAA levels returned to baseline within 60 h postpartum.

Keywords: serum amyloid A, horse, inflammation, pregnancy, CTUP

Equine endometrial concentrations of fluconazole following oral administration

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The objective of this study was to determine the plasma and endometrial concentrations of fluconazole after oral administration to mares. Our hypothesis was that endometrial levels of fluconazole would be maintained above the minimum inhibitory concentration (MIC) of *Candida albicans*.

Group one mares (n=6) in early estrus were administered a single loading dose of 14 mg/kg fluconazole (Glenmark Generics Inc., Mahwah, NJ) via naso-gastric tube. Group two mares (n=3) were administered the loading dose, followed by maintenance doses of 5 mg/kg q 24 hr for six days. Plasma and endometrial biopsy samples were collected at predetermined times and analyzed at the Pharmacology Core Laboratory, Colorado State University using HPLC-MS.

Mean plasma and endometrial fluconazole levels at 24 hrs following loading dose were 8.7 ± 0.2 μg/ml (mean ± SEM) and 12.7 ± 0.3 μg/mg respectively. Fluconazole levels in plasma and endometrium 24 hours following the last maintenance dose were 7.8 μg/ml ± 1.0 and 7.2 ± 2.2 μg/mg, respectively. The published MIC of *Candida albicans* is 0.5 to 8.0 μg/ml. Two of three mares in our maintenance dose maintained uterine levels greater than the MIC (8.0 μg/ml) for *Candida* spp.

In summary, oral administration of fluconazole (5 mg/kg q 24 hrs) will achieve endometrial concentrations near or above the MIC for *C. albicans*.

Keywords: Fluconazole, fungal endometritis, *Candida,* pharmacokinetics

Effects of seminal plasma on frozen-thawed stallion epididymal semen plasma membrane integrity

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Frozen-thawed epididymal sperm of stallions has variable post-thaw motility. Epididymal sperm are collected postmortem or postcastration and therefore are not exposed to the effects of seminal plasma as is ejaculated semen. The objective of this study was to evaluate the effects of seminal plasma plus extender versus extender or seminal plasma alone on epididymal spermatozoa plasma membrane integrity using the hypoosmotic swelling test (HOST). Our hypothesis was that epididymal sperm exposed to seminal plasma would demonstrate better post-thaw membrane integrity as assessed by HOST than epididymal sperm frozen without exposure to seminal plasma. Epididymides were dissected from the testes of mature stallions during the breeding season within one hour of routine castration or euthanasia. Seminal plasma was obtained from a stallion with high fertility and high freezing ability and was centrifuged and stored at -20°C until use. In the first experiment (n=6 stallions) epididymal flushing with a commercial extender (INRA 96, IMV Technologies France, L'Aigle, France) plus 20% seminal plasma (EXSP) was compared to flushing with extender alone (EX). In the second experiment (n=5 stallions), epididymal flushing with extender plus 20% seminal plasma (EXSP) was compared to flushing with seminal plasma alone (SP). Total volume of flushing medium was 20 mL. Semen was extended after 10 min incubation at room temperature to approximately 100 x 10^6 spermatozoa per mL. The extended semen was then centrifuged at 700*g* for 15 min and the semen pellet suspended to 400 x 10^6 spermatozoa per mL in a commercial freezing extender (E-Z Freezin-LE, Animal Reproduction Systems, Chino, CA), loaded in 0.5 mL straws and frozen 2 cm over liquid nitrogen vapor for 20 min. Frozen straws were thawed in a 37°C water bath for 30 sec then incubated at 37°C for 10 min. The HOST was performed using 100 µL of semen added to 1 mL of a 100 mOsm sucrose solution. The percentage of spermatozoa with intact membranes for each treatment was compared using a paired-t test within each experiment after 10 min and 60 min incubation. The results of the first experiment demonstrated that the percentage (± SEM) of spermatozoa with intact (swollen) membranes after epididymal flushing with EXSP was lower than those flushed with EX (53.7 ± 3.4 vs. 58.0 ± 2.7%, p=0.05) at 10 min but a difference was not seen at 60 min (43.7 ± 2.7 vs. 45.7 ± 0.8%, p=0.51). In the second experiment, the percentage of spermatozoa with intact membranes after epididymal flushing with EXSP was not significantly different from those flushed with SP at 10 min (66.8 ± 6.8 vs. 55.6 ± 8.5%, p=0.06) or at 60 min (55.6 ± 8.1 vs. 54.8 ± 8.7%, p=0.9). In conclusion, the addition of seminal plasma to the epididymal flushing extender does not seem to improve post-thaw semen quality of recovered stallion epididymal sperm and in one experiment was slightly detrimental after short incubation. However, additional studies are needed to evaluate if the addition of seminal plasma before processing of epididymal sperm for freezing would improve their fertilizing ability.

Keywords: Hypoosmotic swelling test, cryopreservation, equine, spermatozoa

Isolation and primary cell culture of canine trophoblasts

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Introduction

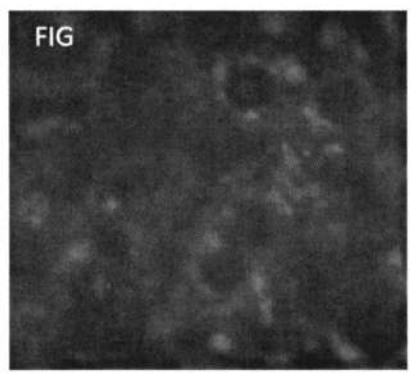
Preeclampsia is a life-threatening condition that affects 5-7% of human pregnancies. Decades of *in vitro* research in this area have been unsuccessful in learning how to prevent it. In preeclampsia, trophoblasts shallowly invade the endometrial endothelium.[1] This defective trophoblast invasion is detrimental to human pregnancy but represents normal endotheliochorial placentation in dogs. The objective of this research was to establish canine trophoblast cell lines to study *in vitro* trophoblast invasion and migration as a model for preeclampsia in humans. Cytokeratin-7 is a type II cytokeratin that positively labels human trophoblasts.[2,3] For this experiment, we hypothesized that cultured canine trophoblasts would also be positive for cytokeratin-7.

Methods

Placentas were removed via hysterotomy from four beagles at 61±1 days from the LH surge (term=65 days). Following methods previously described for isolating human trophoblasts,[4] trophoblasts were isolated using collagenase and trypsin with Percoll density gradient centrifugation. Cells were then cultured in DMEM media (#829415, Gibco-Invitrogen, Carlsbad, CA) at 38°C with 5% CO_2 and grown to 70% confluency on coverslips. Cells were fixed in 70% methanol and expression of cytokeratin-7 (#p103620, DAKO, Carpinteria, CA) was confirmed using fluorescent immunohistochemistry (Alexa Flour 488, #A21202, Invitrogen, Carlsbad, CA). Hoescht 33342 (#H1399, Invitrogen, Carlsbad, CA) was used to count cells.

Results

Cellular morphology was consistent with that of trophoblasts; large polygonal cells arranged in a cobblestone configuration. Occasionally, spherical synctium of cells developed. More than 80% of the cells cultured expressed cytokeratin-7 (Figure).



Conclusion

Using methods for isolating trophoblasts from human placentas, cells isolated from canine placentas had a cellular morphology and immunohistochemistry characteristics consistent with trophoblasts. Future *in vitro* studies using these cell lines will focus on characterizing canine trophoblast invasion and migration.

Keywords: Canine, cytokeratin-7, immunohistochemistry, preeclampsia, trophoblast

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Serum microRNA profiles in cycling mares and mares with granulosa cell tumors

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Granulosa cell tumors (GCTs) are the most common ovarian tumor of the horse and can result in suppression of folliculogenesis, stallion-like behavior, and/or continuous estrus. Circulating microRNAs (miRNAs) in women with ovarian cancer are found to change expression patterns in the presence of disease. The objective of our study was to evaluate serum miRNAs expression via quantitative real-time PCR (qRT-PCR) and determine if levels differed in normal cycling mares vs. mares with GCTs.

Serum samples from nine normal cycling mares (estrus, day of ovulation, and seven days post-ovulation), and 15 mares with histologically confirmed GCTs were evaluated. Relative expression of 383 miRNAs was performed initially profiling five normal mares at all sample time points and five GCTs mares (n=20 samples total). Based on preliminary data a modified list of 127 miRNAs was used to profile remaining samples (normal mare n=12; GCTs mare n=10). Data were analyzed using the comparative Ct method.[1]

Evaluation of serum samples from normal mares and mares with GCTs revealed significant differences in expression level for at least 15 miRNAs. These include: miR-19a, miR-92b, miR-124, miR-302a, miR-432, and miR-501-3p. Results of this study demonstrated changes in the expression of specific circulating miRNAs in mares with GCTs compared to normal cycling mares and additional research is needed to determine the clinical diagnostic potential of these identified findings.

Keywords: Granulosa cell tumor, microRNA, mare

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Effectiveness of etonogestrel implants on estrus suppression in mares

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The objective is to evaluate a human contraceptive implant (Implanon®, Organon labs, Cambridge, UK) as a reliable method to suppress behavioral estrus in mares.

Twenty healthy mares were randomly assigned to 4 groups (n=5). Group 1: control (no treatment), Group 2: one Implanon® subdermal implant (68 mg etonogestrel), Group 3: two implants (136 mg); and Group 4: positive control, 0.044 mg/kg altrenogest (Regu-Mate®, Intervet/Schering Plough Animal Health, Whitehouse Station, NJ) orally daily.

Behavioral estrus was evaluated twice weekly by a blinded observer. Estrous cycles were monitored for three months by weekly progesterone levels and twice weekly transrectal examinations. Interestrus interval (IEI) was determined based on behavioral estrus (teasing scores) and progesterone levels (below 1.0 ng/ml).

Mean IEI (± SEM) per group, based on teasing and progesterone levels respectively, were as follows: Group one: 21.2±0.3 and 21.7±0.4 days; Group two: 34.5±8.2 and 31.4±6.4 days; Group three: 42.7±14.1 and 41±14.4 days; Group four: 111.2±1.3 and 48±0.9 days.

In group four, estrus behavior was suppressed during the entire study period. Group three had an IEI twice as long as group one, which is clinically meaningful; however, no statistical difference was found between groups one, two and three. Group four was different from all other groups ($P<0.05$) based on teasing observations. The IEI determined by teasing and progesterone levels were highly correlated ($r=0.91$).

In conclusion, etonogestrel was not consistently effective for estrus suppression in mares at either dose (68 or 136mg). Future studies with a higher dose would be necessary to determine the efficacy of etonogestrel in suppressing behavioral estrus in mares.

Keywords: Mare, estrus suppression, Implanon, etonogestrel

Susceptibility to delayed uterine clearance after breeding: relationship to endometrial biopsy score and age, and variations between seasons

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The objectives of this study were to 1) compare delayed uterine clearance (DUC) after insemination with age and endometrial biopsy score (Kenney scale), and 2) compare susceptibility to DUC over subsequent breeding seasons. Fifty five mares were inseminated with 10^7 freeze-killed spermatozoa during estrus and evaluated for DUC by transrectal ultrasonography. Delayed uterine clearance was defined as >2 cm intrauterine fluid at 48 h and the presence of fluid at 96 h after insemination. Mares with no fluid retention 48 h after insemination were considered resistant to DUC, and mares with >2 cm of fluid at 48 h, but no fluid at 96 h were classified as "intermediate". A subset of 14 mares was classified for susceptibility to DUC in two subsequent breeding seasons. Comparisons were made using a Chi-square test comparing biopsy score, fluid retention after insemination, and age. Significance was set at $p < 0.05$.

Biopsy score and age were correlated to DUC ($p < 0.001$). In addition, age was correlated to biopsy score ($p < 0.001$) as previously reported. Of the mares examined for susceptibility, 36% (5/14) changed status during subsequent seasons. Three mares changed to a more severe classification (intermediate to susceptible, or resistant to intermediate), while two mares changed to a less severe classification (susceptible to intermediate).

These results suggest that a uterine biopsy may be used in predicting DUC, although some mares may fall into an intermediate classification. In addition, mares may increase or decrease the degree of susceptibility to DUC over subsequent breeding seasons.

Keywords: Endometrial biopsy, intrauterine fluid, equine

Surface architectural anatomy of the penile and preputial epithelia of bulls

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Introduction

A common assumption is that older bulls are more likely to become chronically infected with *Tritrichomonas foetus* and *Campylobacter fetus* subsp. *venerealis*. One theory is that older bulls develop deeper penile and preputial epithelial folds as they age providing a protected, microaerophilic environment suitable for long-term maintenance of *T. foetus* and *C. fetus*. However, multiple case reports exist of young bulls developing chronic venereal infections and no published reports support the theory that older bulls develop deeper penile or preputial epithelial folds. In this study we compared the histological and surface architectural anatomy of penile and preputial epithelia between young and old bulls.

Materials and methods

Twelve Angus bulls were divided into two groups: 1) bulls approximately 2 years of age (Group 1; n=6) and 2) bulls ≥ 5 years of age (Group 2; n=6). Samples of penile and preputial epithelium were collected from three anatomical locations and examined with light and scanning electron microscopy to assess three variables: area of epithelium, total number of epithelial folds, and area contained within the folds. Results were compared between age groups.

Results

No differences ($p>0.05$) were detected between age groups with respect to penile and preputial area of epithelium, total number of epithelial folds, or area contained within the epithelial fold.

Significance

Older bulls do not have thicker penile or preputial epithelium, more epithelial folds, or develop a larger area contained within the fold as they age. Chronic *T. foetus* and *C. fetus* infections in older bulls are unrelated to changes in the surface anatomy of penile or preputial epithelia.

Keywords: Trichomoniasis, penile epithelium, preputial epithelium, epithelial crypts, epithelial folds

Effects of lactoferrin on post-breeding uterine inflammation in the mare

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Post-breeding endometritis is a normal inflammatory reaction of the uterus to sperm that usually subsides within 48 h. However, in some mares, post-breeding inflammation persists for >48 h and negatively affects fertility. Lactoferrin has been shown to play a key role in modulating the inflammatory process in other species. Our objective was to determine the effects of lactoferrin on the post-breeding inflammatory process of the endometrium. Our hypothesis was that lactoferrin would modulate the inflammatory process post-breeding by altering expression of pro-inflammatory cytokines in the endometrium. Six cycling mares were randomly allotted to receive either the control treatment (semen only) or lactoferrin (semen + 1 g lactoferrin) in a cross-over design. When in estrus, mares were inseminated with 1 x 10^9 dead sperm diluted in 50 mL of skim milk based extender with or without 1 g of lactoferrin, and received 2500 IU of human chorionic gonadotropin (hCG) to induce ovulation. Mares were then evaluated daily to determine the time of ovulation and the amount of intrauterine fluid (0 = none; 4 = large). Endometrial culture, cytology, and biopsy were collected approximately at 24 h post-insemination. An endometrial swab was submitted for aerobic culture and the amount of bacterial growth was determined (0 = no growth; 4 = heavy growth). Endometrial cytology was stained with modified Wright Giemsa stain and evaluated to determine the percentage of white blood cells (WBC) in the smear. Endometrial biopsies were immediately frozen and then evaluated by RT-PCR to determine expression of the following genes: IL-1β, IL-6, IL-8, IL-10, and TNF-α. Data were analyzed by Wilcoxon Rank Sum test and significance was set at $P<0.05$. Ovulation was detected in all mares within 48 h of hCG administration. Twenty four hours after insemination, there were no significant differences between control and lactoferrin groups for intrauterine fluid (2.2 vs. 1.7), bacterial growth (1.2 vs. 0.8), and percentage of WBCs (37.3 vs. 21%). However, there was a decrease in the expression of the pro-inflammatory cytokines IL-1 ($P<0.05$) and IL-8 ($P<0.07$). These results are supportive of our hypothesis that lactoferrin modulates the uterine inflammation post-breeding by potentially altering the expression of pro-inflammatory cytokines. Overall, post-breeding inflammatory reaction in the uterus of mares receiving lactoferrin was milder than in control mares. Results from this pilot study are encouraging and warrant further investigation using a larger number of animals.

Keywords: Lactoferrin, endometritis, inflammation, equine, mare

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XY sex reversal in a Quarter Horse mare

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Abnormalities of sexual differentiation in horses are commonly reported, including monosomy X and sex reversal cases.[1-9] These manifest as irresolvable infertility issues with the animal having either ambiguous or female external genitalia and gonads that may not match the karyotype or are hypoplastic.[2,3] A ten year-old Quarter Horse mare presented for breeding management. The client reported that the mare had never been bred and had erratic heat cycles. The external genitalia appeared normal. Transrectal palpation detected a cervix with low tone, small uterine horns and small, firm structures in the areas of the ovaries, which measured 1.2 by 1.6 cm and 1.3 by 1.8 cm on the left and right respectively, using transrectal ultrasonographic evaluation. An intersex condition with gonadal dysgenesis or a prior ovariectomy were considered as potential differential diagnoses. Scar tissue was not present on the flanks nor was it apparent through a vaginal speculum examination, discounting the likelihood of a prior flank laparotomy or colpotomy. Karyotyping revealed that nine cells had a chromosome count of 63,X and thirteen cells had a count of 64, XY. PCR testing with several Y chromosome markers confirmed the presence of a Y chromosome. The SRY gene PCR was negative. Other PCR tests for areas on the Y chromosome outside the SRY gene were positive. FISH was positive for a Y chromosome. Interphase nuclei X and Y probes revealed no XO cells, discounting the previous finding of 63, X cells. It was determined that the mare had a straight XY sex reversal with partial deletion of the SRY gene. The SRY gene initiates the genetic cascade resulting in testicular differentiation and male internal and external reproductive organs.[1,10,11]. Genes encoding for ovarian formation would be activated but an X dosage deficiency would prevent normal ovarian development and function.[10]

Keywords: Intersex, sex reversal, gonadal dysgenesis, genetic abnormality, infertility

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Long-term management of cystic benign prostatic hyperplasia in a valuable breeding dog

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Benign prostate hypertrophy (BPH) is the most common condition of the canine prostate. The recommended treatment for this condition is castration, but in valuable stud dogs, we look at options that allow us to preserve fertility while alleviating clinical signs. This case shows successful management of a dog with Cystic BPH to maintain fertility.

A 3-year old male intact Rottweiler presented with hematuria, dripping blood from the prepuce, and diarrhea. Diagnostics performed included physical examination, abdominal radiographs, complete blood count, serum chemistry, and urinalysis. After urinary tract and coagulation disorders were ruled out, manual semen collection was performed. Physical examination and radiographs revealed a slightly enlarged, non-painful prostate. Prostatic fluid was analyzed with culture and sensitivity, with results ruling out infections and inflammatory conditions. Ciprofloxacin was administered and signs subsided. With later recurrent episodes of tenesmus and ribbon-like stool, ultrasound of the caudal abdomen and prostate was performed. This analysis allowed for the definitive diagnosis of cystic BPH. Ultrasound showed multiple hypoechoic areas within the prostate parenchyma. Additionally, ultrasound evaluation revealed a large paraprostatic cyst in the right caudodorsal abdomen. Approximately 90 mL of yellow-tinged fluid was drained from the paraprostatic cyst, and clinical signs subsided temporarily. Over time, the signs returned and the paraprostatic cyst was removed surgically.

After removal of the paraprostatic cyst, ultrasound was performed at regular intervals to monitor the size of the prostatic cysts within the parenchyma. With continued use of finasteride, the size of the prostate remained static over several years. Several semen collections for shipment or freezing were performed subsequent to surgery and treatment with finasteride, demonstrating continued fertility. Thus, good control of clinical signs related to cystic BPH was achieved, allowing the patient to remain intact for further breeding use.

Keywords: Benign prostatic hyperplasia, prostate disease, cyst, finasteride

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Epididymitis, ampullitis and periorchitis due to an ascending seminal vesiculitis caused by *Pseudomonas aeruginosa* in a stallion

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Internal genital infections in the stallion are uncommon and tend to be localized to the seminal vesicles (seminal vesiculitis) without compromising epididymal or testicular health.[1,3,5-7] This stallion was presented for unilateral scrotal enlargement due to periorchitis and epididymitis caused by ascending seminal vesiculitis. Subsequent spread of the organism resulted in bilateral castration.

The diagnostic approach involved visual examination, manual palpation and ultrasonography to determine that the unilateral scrotal enlargement was due to epididymitis. *Pseudomonas aeruginosa* was cultured from a semen sample. Subsequent evaluation, using endoscopic isolation of fluid from the seminal vesicles, led to diagnosis of unilateral seminal vesiculitis. Treatment included surgical removal of the left testis and epididymis with the intent of salvaging the right testis and epididymis to prolong the stallion's breeding career. During surgery, the stump of the ductus deferens was catheterized and lavaged daily for six days with lactated Ringer's solution and ticarcillin/clavulanate. Two days after removal of the left testis and epididymis, the right testis and epididymis were surgically removed due to peracute epididymitis and periorchitis. The stallion was systemically treated with potassium penicillin, gentamycin, entrofloxacin, flunixin meglumine and sacchromyces capsules.

Pseudomonas aeruginosa can colonize the penis[1] or cause seminal vesiculitis[3,5-7] leading to infertility and reproductive losses.[1,2] Treatment for seminal vesiculitis can be unrewarding, but fertility can be maintained using semen extenders.[3,5] *P. aeruginosa* is the most common bacterial isolate from the internal genital tract[4] and may be difficult to eliminate completely.[5] This is an example of seminal vesiculitis that initially affected only one testis and epididymitis, but subsequently spread to the contralateral side resulting in bilateral castration. Ascending seminal vesiculitis has not been previously reported and should be considered as a potential threat to the health of the testes and epididymides.

Keywords: *Pseudomonas aeruginosa*, periorchitis, epididymitis, orchidectomy, ultrasonography

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Neonatal isoerythrolysis and alloimmune neutropenia in the foal of a primiparous mare

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Isoerythrolysis is the most common cause of jaundice and hemolytic anemia in equine neonates. Ingestion of offending colostral antibodies produced following sensitization to incompatible red cell antigens induces this potentially lethal hemolytic event. Initial exposure usually occurs during pregnancies in which the foal has inherited incompatible antigens from the sire. Thus, foals of pluriparous mares are at risk.

A five day old colt of a primiparous Thoroughbred mare presented severely anemic and leukopenic. The colt had been non-responsive at birth requiring oxygen supplementation and plasma infusion. Marginal improvement was followed by rapid deterioration and an acute episode of dyspnea. Upon presentation the colt was depressed, tachycardic and tachypnic with icteric mucous membranes and a PCV of 11%. A packed red blood cell transfusion was performed and prophylactic antibiotics initiated. Following transfusion a complete blood count (CBC) showed neutropenia and anemia, although hematocrit was improved. Serum chemistry showed hypoproteinemia, hypoalbuminemia and hyperbilirubinemia. Clinical diagnosis of neonatal isoerythrolysis was based on anemia, positive minor cross-match and positive Coomb's test. Abdominal and thoracic ultrasound and blood culture ruled out regional infection and septicemia as causes for continued neutropenia. Positive agglutination of neutrophils with mare's serum supported diagnosis of alloimmune neutropenia later confirmed via flow cytometry. During hospitalization CBC's were repeated regularly. Neupogen was administered as needed until neutrophil counts stabilized within normal reference ranges.

Neonatal isoerythrolysis has not been reported in the foal of a primiparous mare and there is only one reported case of alloimmune neonatal neutropenia. This case is a unique presentation of these conditions concurrently. Presumably, sensitization to foreign red blood cell and neutrophil antigens occurred simultaneously via a single exposure event. Previous blood transfusion, normal late gestation placental microhemorrhage or previous conception with subsequent abortion are potential scenarios which may have lead to colostral antibody production in this case.

Keywords: Neonatal isoerythrolysis, alloimmune neutropenia

Suggested reading

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Ovarian abscess in a maiden mare

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A 17 year-old maiden Quarter Horse mare was referred to Texas A&M University after a one week history of intermittent colic and pyrexia (102-104°C). She was subsequently diagnosed with a unilateral ovarian abscess. Colic and pyrexia tend to be associated with gastrointestinal distress in the horse but may also result from ovarian pathology.[1,2]

At admission, the mare had hyperfibrinogenemia, neutrophilia, globulinemia and was bright, alert, and normothermic (100.1°F). Transabdominal ultrasonography and abdominocentesis identified an increased amount of orange-opaque peritoneal fluid (923,000 WBC/μL [85% neutrophils]). Initial treatment included antibiotics, anti-inflammatory and anti-ulcer medication. The following day, manual and ultrasonographic evaluation of the reproductive tract determined the mare had poor perineal conformation, pneumouterus and an enlarged right ovary with two fluid-filled cavities (~5 and 6 cm in diameter). The right ovary was removed and histopathologic diagnosis determined it was an anovulatory hemorrhagic follicle with an adjacent ovarian abscess.

The differential diagnosis for an "enlarged" ovary in the mare includes neoplasia, hematoma, transitional ovary, and abscess.[3-5] Ovarian abscess is uncommon, but tends to be associated with a fever of unknown origin and colic similar to the mare in this case.[6] Ovarian abscesses may result from rough handling of the ovary, ovariocentesis, hematogenous spread of bacteria, or migration of strongyle larva.[2,6-8] In this case, the mare had never been bred or any history of ovarian manipulation, but did have a potential source of infection (pneumouterus). Although relatively common in cattle,[9] ascending infection from the uterus to the oviduct and ovary is rare in the mare because of the presence of the oviductal papilla.[10] An ovarian abscess should be considered in the differential diagnosis for a mare with intermittent colic and fever of unknown origin.

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Normal parturition after unilateral ovariectomy and uterine leiomyoma removal in a Thoroughbred mare

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Uterine tumors are rare in horses. Leiomyomas, tumors arising from smooth muscle tissue, are the most commonly diagnosed equine uterine neoplasm.[1,2] Leiomyomas are usually benign and pedunculated, but may also present as an intramural mass.[2,3] Leiomyomas are sometimes associated with infertility.[4]

A ten year old Thoroughbred mare presented to the University of Florida College of Veterinary Medicine for evaluation because of a two year history of infertility. Rectal palpation and ultrasonography revealed a 5.23 cm, well-circumscribed mass located at the tip of the left uterine horn without involvement of the left ovary. This was confirmed by hysteroscopy. Doppler examination revealed the presence of three large vessels associated with the mass. Manually assisted laparoscopic ovariectomy and left uterine horn mass removal (approximately one-third of the horn) were performed under standing sedation. Histopathologic evaluation of tissues revealed fusiform cells forming broad interlacing fascicles with a fibrovascular connective tissue stroma, consistent with a leiomyoma. There was no involvement of the papilla of the oviduct. The mare had an uneventful recovery. She was bred by live cover and conceived twins as a result of the first mating. After successful manual reduction of one embryonic vesicle, the mare carried the remaining pregnancy to 338 days gestation. The mare delivered a healthy male foal with an estimated weight of 65 kg. Parturition was uneventful and placental evaluation revealed pregnancy confined to one horn.

Uterine leiomyomas often cause infertility in the mare due to the obstructive nature of masses and interference with maternal recognition of pregnancy. Uterine biopsy is a definitive diagnostic method. Surgical removal of the mass(es), which may involve unilateral ovariectomy and partial hysterectomy, is recommended.[4] This case documents successful maintenance of pregnancy after leiomyoma removal including one-third of a uterine horn.

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The use of ceftiofur sodium in the extension and cooled storage of equine semen: its effects on motion characteristics, pH, and osmolality

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The purpose of the current study was to determine and compare the effect of varying antimicrobial concentrations in a semen extender on equine spermatozoal motion characteristics, semen osmolality, and semen pH. A total of 13 ejaculates from three stallions were examined. Each ejaculate was divided and extended in a skim milk-glucose based semen extender (Har-Vet™ Semen Extender, Har-Vet™, Spring Valley, WI) without antibiotic (control) or that contained one of seven different antimicrobial drugs: ceftiofur sodium (Naxcel®, Pfizer Inc., New York, NY) at 250 µg/mL; 500 µg/mL; 1,000 µg/mL; 2,500 µg/mL (CEFT2500); combination of amikacin sulfate (1,000 µg/mL) and potassium penicillin G (1,000 IU/mL) (AMKPCN); gentamicin sulfate at 1,000 µg/mL (GENT); and ticarcillin disodium at 1,000 µg/mL (TICAR). Extended semen was cooled and stored at approximately 5°C. Motility measures using computer-assisted semen analysis (Sperm Vision® CASA, Minitube®, Verona, WI), pH measurements (Accumet AB15, Fisher Scientific Inc., Hanover Park, IL), and osmolality measurements (5010 Osmette III™, Precision Systems Inc., Natick, MA) were performed at 0, 24, and 48 h after collection. A statistical software program (SAS 9.1, SAS Inc., Cary, NC) was used for all statistical analyses. A mixed model analysis of variance was used to determine the main effect of antimicrobial group on measured parameters. Ceftiofur sodium had a dose-dependent effect on curvilinear distance, curvilinear velocity, and the amplitude of lateral head displacement, with higher concentrations of ceftiofur (1,000 and 2,500 µg/mL) increasing these spermatozoal motility measures in comparison to control (F test, $P<0.05$; post-test comparison, $P<0.0071$). There was also an effect of antimicrobial group on straightness of motility (F test, $P<0.05$) with higher concentrations of ceftiofur decreasing values in comparison to control (post-test comparison, $P<0.028$). Stepwise multivariable regression analysis revealed pH was the strongest indicator for the increased motion characteristics, but it was only a minor predictor ($R^2 \leq 4.0\%$). There was also a significant effect of antimicrobial group on extender pH, with pH being decreased in the CEFT2500, AMKPCN, and TICAR groups, and increased in the GENT group compared to control. There was no significant effect of antimicrobial group on extender osmolality. The changes in motion characteristics associated with higher extender concentrations of ceftiofur are consistent with a dose-dependent tendency toward spermatozoal hyperactivation by ceftiofur through an unidentified mechanism. Additional studies appear indicated to evaluate the efficacy of ceftiofur sodium in the extension and cooled storage of stallion semen and its relationship to hyperactivation of equine spermatozoa.

Keywords: Spermatozoa, ceftiofur sodium, motility, pH, osmolality

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Acupuncture as an alternative treatment for persistent breeding induced endometritis in mares

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All mares, when bred or inseminated, respond to the ejaculate with an inflammatory reaction. Many subfertile mares are unable to clear this normal post-breeding inflammation, and suffer from persistent post-breeding endometritis (PPBE). Acupuncture is an alternative therapy which is rapidly becoming commonplace in equine veterinary practice. Traditional Chinese Veterinary Medicine sources claim that certain acupuncture points can be used to affect cervical tone, uterine contractility and local immune function in mares suffering from PPBE. Unfortunately, much of this evidence consists of anecdotal reports, or is hypothetical based on evidence from studies conducted in humans or other species, and reliable data are lacking.

We undertook to critically evaluate the effect of acupuncture on uterine clearance in mares susceptible to PPBE in a series of clinical trials. The pilot study reported here utilized 12 mares previously confirmed to be susceptible to PPBE. The mares were randomized to one of two groups; oxytocin (positive control), or dry needle acupuncture at GV 1, LIV 8, GB21, SP 6, SP 9, LI 4, BL 22, BL 30, BL 67 bilaterally and Baihui. Estrus was induced with dinoprost tromethamine (5 mg SQ). When estrus was confirmed (follicle ≥32mm, ≥ Grade II endometrial edema and behavioral estrus present), 2500 IU human chorionic gonadotropin (hCG) was administered IV. Twenty-four hours after hCG administration 100ml 0.9% sterile saline was infused into the uterus. Prior to the infusion, ultrasonography was performed to evaluate free intraluminal uterine fluid and samples were collected using a sterile double guarded uterine swab and evaluated for exfoliative endometrial cytology. Four hours post-infusion, cytology and ultrasonographic evaluation of free intraluminal fluid were repeated. After sampling, oxytocin 30 IU IV (n = 6), or acupuncture (n = 6) was administered. Acupuncture duration was 20 minutes with the exception of BL67, which was bled. Twenty four hours post-infusion, cytology and ultrasonography were repeated. Those mares with no free intraluminal uterine fluid were not treated further. Any mare with residual free intraluminal uterine fluid underwent a second treatment. These mares were examined and sampled again at 48 hours post-infusion. To ensure blinding, experimental treatments were administered by one team (DS, STM), and examinations and sample collections were performed by another (NT, NHC, AMD).

Mares treated with acupuncture were 4.00 times more likely than mares treated with oxytocin to have a negative endometrial cytology at 24 hrs post-infusion with sterile saline (Chi Square, OR 4.00, $p = 0.046$). There were no significant differences between treatment groups in cytology score at 0, 4 and 48 hours post-infusion. There were no significant differences between treatment groups in free intraluminal uterine fluid scores at any sampling times in this study. Acupuncture may benefit some mares with PPBE, but more research is required.

Keywords: Endometritis, acupuncture, clearance, mares, uterus

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Streptococcus equi subsp. *zooepidemicus* isolates from infectious endometritis belong to a distinct genetic group as assessed by pulsed-field gel electrophoresis

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Streptococcus equi subspecies *zooepidemicus* (*S. zooepidemicus*) is the most frequently isolated pathogen from the uterus of the mare. *S. zooepidemicus* is an opportunistic pathogen and a part of the resident flora of the caudal reproductive tract. The aim of the study was to genotype and compare *S. zooepidemicus* strains of from the uterus of mares with endometritis with isolates from the vagina and fossa clitoridis using pulsed-field gel electrophoresis (PFGE).

The mares (n=29) included in the study were of different breeds and aged three to 25 years. Mares with (n=18) or without (n=11) clinical signs of endometritis were included. Uterine samples were collected using a guarded endometrial biopsy punch (Equi-vet®, Kruuse, Marslev, Denmark). A double-guarded swab (Kruuse) was used to recover samples from the cranial vagina and samples from the fossa clitoridis were collected using a sterile swab (BBL™CultureSwab™, BD, Franklin Lakes, NJ). Samples were incubated on blood agar at 37°C for 24 hours. Only pure cultures (≥90% of all colonies) from the uterine and vaginal swabs were included in this study. If *S. zooepidemicus* was present, up to three colonies were selected from each anatomical location (maximum nine samples per mare). The bacterial isolates were characterized by PFGE using the *sma*I restriction enzyme (New England Biolabs Inc., Ipswich, MA).

In 12 mares *S.* zooepidemicus was isolated from the endometrium. A total of 88 *S.* zooepidemicus isolates were analyzed, 31 from the endometrium, 26 from the cranial vagina, and 31 isolaes from the fossa clitoris. Analysis of the individual banding patterns demonstrated a genetic similarity of the *S. zooepidemicus* isolates obtained from infectious endometritis, which was different from the group of isolates obtained from the caudal reproductive tract. In conclusion the study indicates that a particular genetically distinct group of *S. zooepidemicus* is associated infectious endometritis in the mare.

Keywords: Endometritis, mare, *Streptococcus*, pulsed-field gel electrophoresis

Endometrial gene expression of inflammatory cytokines and serum amyloid A in mares resistant or susceptible to persistent endometritis

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The objective of the study was to evaluate the endometrial gene expression of inflammatory cytokines (IL-1β, IL-1RA, IL-6, IL-8, IL-10, TNFα) and serum amyloid A (SAA) in response to inoculation of 10^5 CFU *Escherichia coli* in mares resistant or susceptible to persistent endometritis, respectively.

Mares were classified as resistant or susceptible based on their clinical response to uterine inflammation and endometrial quality. Endometrial biopsies were recovered 3, 12, 24 and 72 hours (h) post-inoculation. Relative gene expression analyses were performed by quantitative real-time PCR using SYBR green and specific primers.

Resistant mares initially (3 h) showed an up-regulated endometrial gene expression of IL-1β, IL-6, and IL-8 ($p<0.05$) after *E. coli* inoculation compared to estrous baseline levels. The susceptible mares showed increased gene expression of IL-6 and IL-1RA ($p<0.05$) 3 h after bacterial challenge. Susceptible mares showed a sustained and prolonged inflammatory response with increased gene expression levels of IL-1β ($p<0.01$), IL-8 ($p<0.01$) and IL-1RA ($p<0.001$) at the end of the study period (72 h) compared to resistant mares. Endometrial mRNA transcripts of IL-1β and IL-1RA were significantly elevated in mares with heavy uterine bacterial growth ($p<0.05$).

The current investigation suggests that endometrial mRNA transcripts of pro-inflammatory cytokines as a response to endometritis are finely regulated in resistant mares, with an initial high expression level followed by normalization within a short period of time. Susceptible mares had a prolonged expression of pro-inflammatory cytokines, supporting the hypothesis that an unbalanced endometrial gene expression of inflammatory cytokines might play an important role in the pathogenesis of persistent endometritis.

Keywords: Infectious endometritis, endometrial inflammatory response

Decreased competence of uterine response to uterine lavage with lactated Ringer's solution in diestrus and clenbuterol treated mares

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Uterine lavage with various solutions causes acute endometritis in the mare varying in duration and severity. The objective of this study was to assess the influence of estrous cycle stages and uterine motility on the uterine response after a uterine lavage (UL) with 2 L of lactated Ringer's solution. It was hypothesized that such a UL induces a transient acute endometritis with a greater intensity in diestrus, which could be partly explained by decreased uterine motility. Each of the same five mares (mean age 11 y, SD=1.9) received three successive ULs at four month intervals: the first was performed 7 d after ovulation (diestrus group or D), the second and third during estrus with a 35 mm follicle after hCG injection, with tocolytic treatment (1.5 mg IV;Ventipulmin, Boehringer Ingelheim, Burlington, ON, Canada) 40 h after the third UL (estrus group or E and estrus treated group or ETx). Just before each UL (day 0) and on days 1, 2 and 4, a standard uterine culture was taken, mean of neutrophils from the cytobrush-collected specimens (Fisher Scientific, Whitby, ON, Canada) were counted microscopically in 10 fields (400 x magnification). From day 0 to day 4, blood was also taken for progesterone assay and complete genital examination was performed including cervical flaccidity scoring, ultrasound grading or measures of endometrial edema, diameters of uterine body and horns, intraluminal fluid and air (Aloka 500, Imago, Vaudreuil, QC, Canada). Means of log of neutrophil counts, means of uterine diameters, cervical flaccidity, presence of fluid and progesterone concentrations were analyzed using a linear regression model with repeated measures; and the effects of the day and of the group on bacteriological and edema scores, and presence of air were analyzed with the Cochran-Mantel-Haensel test (SAS 9.1 software, Cary, NC).

Cervical flaccidity, endometrial edema and uterine diameters were significantly different between groups D and E at day 0, no significant difference between these parameters was observed by day 2. Prevalence of air within the uterus increased ($p=0.008$) earlier in group D (at day 1) versus E (day 2), and by day 2 did not differ significantly between the three groups. Progesterone profiles were significantly higher in group D versus E until day 3. Bacterial growth scores (from 0 to 5, positive by 2) increased at day 2 ($p=0.04$) in group D (mean=3.4, SD=1.8) versus E (mean=1, SD=0.7). Neutrophil counts were higher ($p=0.0002$) at day 2 in group D (mean=58.4, SD=90.8) versus E (mean=1.8, SD=1.6). The uteri of ETx mares presented an intermediate response when compared to group D: no significant difference of neutrophil counts and bacterial scores at day 2, of progesterone concentrationss by day 3, increase in cervix flaccidity ($p=0.04$) and decrease in endometrial edema ($p=0.03$) at day 4. Depressed diestral mechanical uterine clearance, caused by a closed cervix and decreased uterine contractions, very probably plays a role in neutrophil and bacteria clearing from the lumen. Exfoliative endometrial cytology indicates not only the inflammatory intensity but also the uterine clearance function.

Keywords: Equine endometritis, exfoliative cytology, uterine lavage, uterine clearance, clenbuterol

Development of a broad range qPCR assay to detect and identify fungal DNA in equine endometrial samples

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The aim of this study was to develop a broad range 28S rDNA qPCR assay for detection of fungal pathogens in the equine uterus.

The qPCR assay was optimized using ATCC fungal organisms. Samples were heat-shocked, and then the DNA extracted, amplified, detected, and the sequence analysis of amplicons performed.

Identification of fungal organisms was compared between traditional biochemical analysis and the qPCR assay from 12 clinical uterine samples. Samples were analyzed in the qPCR assay on three different occasions to evaluate variation in the assay.

The primers had a detection limit of 2×10^{-14} g of DNA/μL. Fungal DNA was detected in all 12 clinical samples using the qPCR assay. Identification of a fungal organism was achieved in 94% (34/36) of amplicon submissions. In contrast, positive identification was available for only 75% (8/12) of samples submitted for standard microbial culture. Biochemical identification differed from DNA sequencing identification in two of eight samples for which an organism was reported.

The development of the qPCR technique to detect fungal DNA may allow for rapid identification of fungal endometritis in mares when traditional culture and biochemical classification have failed.

Keywords: Mare, fungal, endometritis, diagnosis, PCR

Detectable differences in the endometrial microbiome between normal and susceptible mares using metagenomic profiling and conventional bacterial culture

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The purpose of this study is to characterize the endometrial microbiome of subfertile mares utilizing 16S rDNA-based metagenomic analysis in order to identify microbes which are otherwise undetectable with conventional diagnostics. Our hypothesis is bacteria that are undetectable by routine culture play a role in the sustainability of the microbiome of the equine endometrium of infertile mares. Mares (n=30) were determined to be subfertile based on the following clinical criteria: inseminated two or more times with no pregnancy in one breeding season, history of two or more years of reproductive failure, two or more unsuccessful embryo recovery attempts during consecutive cycles, two or more unsuccessful pregnancies in recipient mare, 2 cm or greater fluid accumulation in the uterus during any stage of estrus, diestral fluid accumulation, and any fluid accumulation 72 hours post-breeding. A double guarded endometrial sample was first obtained from the uterus, placed in a sterile tube with 10% Tris-EDTA buffer, snap frozen to -196°C and maintained in liquid nitrogen for later RNA analysis. A guarded endometrial biopsy was then obtained for histology, culture and DNA analysis. Using sterile technique, approximately 3 mm of endometrium was excised from the fresh endometrial biopsy for submission for aerobic culture. The remaining one centimeter of tissue was fixed in 10% buffered formalin saline submitted for histology and classified by the standard Kenney-Doig grading system. Histology grade was included to support correlations between metagenomic analysis in determining presence of true microbes versus contamination. DNA was extracted from endometrium embedded in paraffin and a fragment of the eubacterial 16S rDNA gene was PCR amplified using conserved primers. The PCR products were then cloned and sequence analyzed using an ABI3130 DNA analyzer (AME Bioscience, Toroed, Norway). Resulting DNA sequences were compared to publicly available databases using BLAST and various other metagenomic analysis tools.

Preliminary results of sequence analysis revealed a population of anaerobic bacteria which were undetectable by conventional aerobic culture. RNA analysis of double guarded culture swabs looking at gene expression of endometrial biome are still pending. Further samples will be obtained from fertile mares in spring of 2011 to determine if metagenomic communities differ within these two populations. This avenue of research is being pursued to help answer questions directed at bacterial gene expression in fertile mares compared to sub- or infertile mares and the primary and secondary role of unculturable bacteria if any in the maintenance of unfavorable endometrial microbiome.

Keywords: Metogenomics, endometrium, equine, 16s DNA gene, subfertile

Aknowledgements

The mares in this study were provided by local private practitioners; Dr Bryant Craig, Dr. Bryan Carroll, and Dr. Dee Gragg.

Effects of porcine zona pellucida (pZP) vaccination with SpayVac® using three different adjuvants on ovarian activity in mares

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Introduction

Contraception can be provided to wild horses in a humane, economically feasible, and publicly acceptable manner. Currently, pZP immunocontraception has the greatest potential to control fertility because it is easy to administer, is safe, can provide multi-year efficacy, and has minimal side effects. SpayVac® (ImmunoVaccine Technologies, Inc. [IVT], Halifax, Canada) is the only single-dose pZP vaccine with proven multi-year contraceptive efficacy, which makes it practical and economical for broad-scale application in the field. Because the pZP antigen is a poor immunogen, vaccine effectiveness depends upon choice of adjuvant and formulation. The objective of this study was to compare the effect of vaccinating with pZP formulated as SpayVac® containing one of three adjuvants (proprietary IVT adjuvant, non-aqueous MFA [modified Freund's adjuvant], and aqueous emulsion MFA) on ovarian activity in mares. Our hypothesis was that, irrespective of adjuvant, SpayVac® vaccination would not affect ovarian activity in mares.

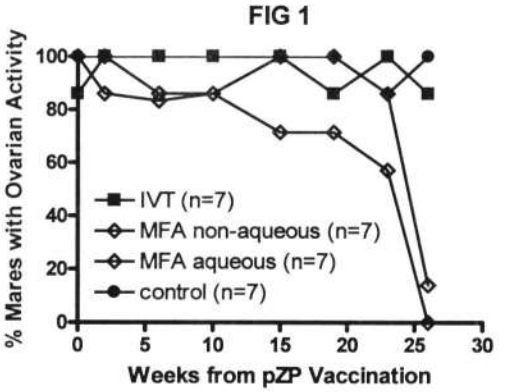
Materials and methods

In March 2010, mares received one (2-ml) intramuscular injection in the neck of SpayVac® formulated with IVT adjuvant (n=7), non-aqueous MFA (n=7), aqueous emulsion MFA (n=7), or placebo controls (n=7). For 26 weeks, ovarian activity was determined by transrectal ultrasonography and palpation. Follicle size was recorded as diameter in mm. The percent of mares with ovarian activity (follicles ≥10 mm with or without a corpus luteum) was analyzed by the non-parametric Wilcoxon rank sum test (GraphPad Prism®, GraphPad Software, La Jolla, CA). Significance was defined as $p<0.05$.

Results

Placebo controls and mares receiving SpayVac® with IVT adjuvant continued to cycle normally throughout the trial (FIG 1). However, by the end of the study, mares treated with SpayVac® containing non-aqueous MFA or aqueous emulsion MFA had significantly smaller ovaries and fewer follicles that could be distinguished ultrasonographically ($p< 0.001$).

FIG 1



Conclusion

SpayVac® in MFA (either non-aqueous or aqueous emulsion) disrupts ovarian activity in mares 26 weeks after vaccination. It is not known if this effect is reversible. The mechanism is under investigation.

Keywords: Horse, immunocontraception, ovarian activity, pZP, SpayVac®

Pro-inflammatory cytokine gene expression in endometrial cytobrush samples harvested from cows with and without subclinical endometritis

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A detailed understanding of the postpartum cow's uterine immune responses is lacking due to difficulties in obtaining endometrial tissue. Our objectives were to develop a minimally invasive cytobrush technique to collect endometrial cells in postpartum cows (28 to 41 days in milk; DIM) for the isolation of mRNA, and to characterize the expression of key regulatory cytokines including: IL-6, IL-8 and TNFα. Cows without clinical signs of endometritis were subdivided into those with a negative or positive (defined as >18% neutrophils) endometrial cytobrush cytology. Thirty Holstein cows were sampled from six commercial dairies. A modified cytobrush was double guarded and advanced into the cervix, passed through the cervical canal and advanced into the uterine body, rotated 360 degrees and retracted into its sheath before removal from the reproductive tract. The cytobrush was then gently rolled onto a clean slide for cytology, and then transferred to a tube with 1 ml Trizol® reagent (Invitrogen, Carlsbad, CA) and stored at -80°C until mRNA isolation. Slides were air-dried and stained with modified Wright Giemsa stain. Each slide was examined at 400x magnification, and three differential counts of >100 cells were averaged for percentage of neutrophils. Total RNA from cytobrush samples was extracted, isolated and reverse transcribed to make cDNA which was used to perform real time (RT)-qPCR analysis of IL6, IL8, TNFα and β-Actin gene expression. Validated gene primers were used to amplify cDNA targets using a two-step qRT-PCR kit with SYBR® Green, in a real time thermocycler). The RT-qPCR amplification data were normalized to β-Actin (ΔCT). Variables were percentage of neutrophils in cytobrush cytology and quantified mRNA expression levels for IL-6, IL-8 and TNF α. Data were analyzed using STATA version 10. Twelve cows were categorized as endometritis positive and 18 were categorized as endometritis negative. Cytobrush sampling provided sufficient material for endometrial mRNA extraction (mean 0.96 μg total RNA per sample). Cytokine expression varied with IL-6 showing a 30-fold higher expression level (P=0.01) and IL-8 showing >50-fold higher expression level (P=0.0001) in subclinical endometritis positive versus negative cows. The TNFα mRNA expression level in subclinical endometritis positive cows was 20-fold higher (P=0.001) versus the disease-negative cows. Regression analysis between mRNA expression levels (ΔCT) of cytokines and percentage neutrophils in endometritis positive cows showed that for every threshold cycle increase in IL-8 expression, the number of neutrophils decreased by 3.3% (P= 0.00001). Similarly, for IL-6 and TNF-α, the number of neutrophils counted in endometritis positive cows decreased by 2.3% (P=0.015) and 2.4% (P=0.054) respectively. In conclusion, the cytobrush technique can be used to obtain sufficient endometrial material for both cytology and RNA extraction and the current analysis confirms that cytology accurately reflects endometrial inflammation.

Keywords: Cytobrush, postpartum endometritis, inflammation, mRNA expression

Reduction of testis size in postnatal pigs using a depot progestin

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Surgical castration is a practice chosen by most swine producers because it is relatively easy to perform, inexpensive and effective in reducing boar odor in pork. It can also alleviate potential behavioral problems associated with raising intact males. The practice has come under scrutiny in the past 20 years as animal welfare concerns are being raised regarding the pain associated with surgical removal of the testes. The objective of the study was to investigate chemical castration of pigs as an alternative to the regular surgical castration procedure that has historically been used to prevent boar taint in the resultant pork products. The primary goal was to determine if a single exposure of a subcutaneous administration of a depot progestin (medroxyprogesterone acetate; Depo Provera®, Pfizer Inc., New York, NY) in postnatal pigs will eliminate Leydig cell proliferation and production of high testosterone levels that can result in boar taint.

Crossbred boars (n=15) were administered a single injection of depot progestin at ten weeks of age and monitored weekly and biweekly for serum testosterone and androstenedione levels as well as testis size measured by calipers. Control boars were treated with a saline injection at 10 weeks of age. Animals were sacrificed at 180 days of age and fat samples obtained for further analysis. Data were analyzed using a repeated measures MANOVA using SAS (SAS 9.1, SAS Inc., Cary, NC). The results of this project reveal that testis size of postnatal boars treated with depot progestin is significantly decreased ($P < 0.001$) over time compared to control boars. The body weights of the treated and control groups were similar ($P > 0.05$) throughout the entire study. The reduction in testis size was not such that personnel could not tell that it was an intact male. Steroid hormone assays (testosterone and adrostenedione) indicated that levels of both hormones were decreased significantly ($P < 0.05$) for 4-6 weeks post-treatment, but then returned to pre-treatment levels and were comparable to control boars. These results confirm that synthetic progesterone can suppress testosterone production and decrease testis size of treated animals, but this effect is not sustained until the normal time of 22-26 weeks of age for slaughter of these animals in the U.S. Further studies need to be performed in order to evaluate the correct timing and dosage for treatment of postnatal pigs with depot progestin as it was believed that there was a dilution effect of the drug as the animals grew.

Keywords: Swine, depot, progestin, castration, medroxyprogesterone acetate.

Acknowledgement

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Evaluation of stress response to electroejaculation in anesthetized alpacas (*Vicuña pacos*)

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Semen collection, evaluation and preservation are important aspects of production animal management. In alpacas, semen collection with an artificial vagina is not always practical. Semen is collected by electroejaculation (EE) under field conditions. Concerns have been raised regarding levels of stress induced by this procedure. The objective of this experiment was to determine if EE would result in significant changes in serum cortisol, respiratory rate, and heart rate in adult male alpacas indicative of a stress response. Males (n=8), aged two to seven years, were used in a randomized crossover design with two treatments (anesthesia with EE and anesthesia alone). All males were anesthetized using a combination of ketamine HCl, xylazine HCl and butorphanol tartrate (KBX). Electroejaculation was performed with a Model 304 EE and #4 probe (P-T Electronics. Boring, OR). Heart rate, respiratory rate, and serum cortisol concentrations were recorded at five time points (2 h pre-induction, 10 min pre-induction, during EE, upon recovery, and 30 min post-recovery). Two males were excluded from analysis because of poor response to anesthesia. Serum cortisol was assessed by radioimmunoassay (Coat-A-Count Cortisol [TKCO1], Siemens Healthcare Diagnostics, Los Angeles, CA). Data were analyzed using repeated measures ANOVA at the level of $p < 0.05$. Overall, the heart rate, respiratory rate, and serum cortisol concentrations were not statistically different between the two treatments ($p > 0.05$). These data suggest that EE does not increase the stress response under the common field anesthesia technique (KXB). Further studies are in progress to determine the reliability of this protocol in obtaining semen samples for reproductive evaluations.

Keywords: Semen collection, serum cortisol, respiratory rate, heart rate, male alpacas

Preliminary results of estrus synchronization and timed artificial insemination in domesticated reindeer (*Rangifer tarandus*)

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Artificial insemination (AI) has been adapted for use in the conservation of threatened and endangered species, but its use for the genetic management of small populations of deer, antelope and other non-domestic bovids has met numerous challenges and limited success. Attempts at AI in reindeer have been met with mixed results. The objective of this study was to determine if estrus synchronization and timed AI would lead to acceptable pregnancy results.

Six adult non-mated female reindeer ages two to six years were selected and housed separately from male animals prior to initiation of the fall breeding season. Females were placed under controlled photoperiod (10 hours of light; 14 hours of darkness) for three weeks beginning the last week of August. Females were synchronized with a 14 day ovine controlled drug release device (CIDR), and received an injection of cloprostenol (250 μg, im) at the time of CIDR removal.

Forty four hours following CIDR removal, timed AI was performed using two 0.5-ml straws of frozen- thawed semen. Each straw contained a minimum of 10×10^6 progressively motile sperm. A GnRH injection (100 mcg im) was also given at the time of AI. A speculum-guided standard French-style AI gun was used to perform transcervical insemination. With this procedure, a clear speculum was inserted into the vaginal canal and the os cervix located visually with the aid of a fiber-optic lightsource connected to the speculum. The AI gun (OD = 4 mm) was then inserted into the os cervix and manipulated through the cervical canal. Semen deposition occurred at the internal os, or just within the uterine body in all females.

At 48 days after AI, four of the six females (66%) were diagnosed pregnant by transrectal ultrasonography. Blood samples were also drawn and submitted for level determination of pregnancy specific protein (PSPB) through bioPRYN.® (BioTracking LLC, Moscow, ID). Samples with an optical density greater than 0.631 were consistent with pregnancy. Pregnancy specific protein results agreed with the transrectal ultrasonography in all females.

In conclusion, timed artificial insemination using frozen-thawed semen in reindeer can be performed in an on the farm setting using this simple synchronization and AI technique, however, per cycle pregnancy rates should be improved to match natural mating results. Additional data are being collected with larger groups of females comparing timed AI vs. heat detection as well as other insemination techniques.

Keywords: Reindeer, estrous synchronization, artificial insemination

Effects of a canine gonadotropin releasing hormone (GnRH) vaccination on male reindeer

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Introduction

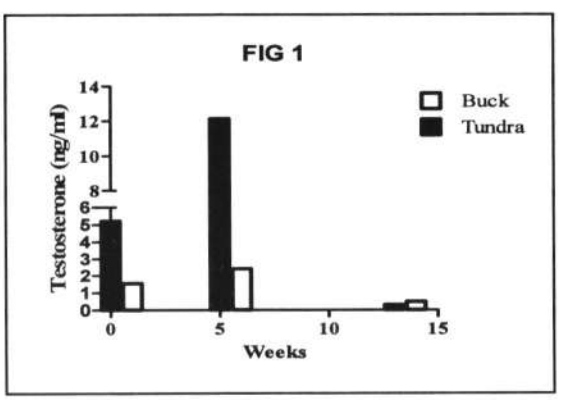
Rutting is a behavior seen in male Norwegian reindeer (*Rangifer tarandus*) and includes losing weight, sparring between bulls, increased aggression toward humans, rubbing on trees, urinating on legs, protecting the harem, and establishing hierarchy.[1] Photoperiod is the main contributor to rut as a decrease in day length increases melatonin secretion, which in this species increases gonadotropin releasing hormone (GnRH), luteinizing hormone (LH) and testosterone secretion, resulting in an increase of rutting behavior.[1] Immunization against GnRH causes declines in testosterone concentrations in many mammals. The aim of this study was to determine if a canine GnRH vaccine manufactured in the U.S. would decrease serum testosterone concentrations and rutting behavior in reindeer. The hypothesis was the GnRH vaccination in male reindeer would decrease testosterone and rutting behavior.

Materials and methods

Two mature (6 and 11 years) intact male reindeer (latitude 39°N) received a series of three 600-µg im injections of Canine Gonadotropin Releasing Factor Immunotherapeutic vaccine (Pfizer Animal Health, Exton, PA) at 0, 5 and 13 weeks (corresponding to 8/20/10, 9/24/10, and 11/19/10). Jugular venous samples were collected prior to vaccination. Serum testosterone was measured using chemilluminescence. All of the samples were tested within one assay. Rutting behavior was assessed by questioning the owner.

Results

Testosterone concentration peaked during the rutting season (5 weeks after initial vaccination) and then decreased to basal levels (<0.5 ng/ml) 13 weeks after initial vaccination (Figure). Two weeks after the second dose, rutting behavior decreased and the antlers were cast, which was five months earlier than previous years for antler casting.



Conclusion

Antler casting occurs soon after surgical castration in cervids.[2] The behavioral and physical responses following GnRH vaccination support the hypothesis that immunocontraception decreases serum testosterone in reindeer.

Keywords: GnRH, rutting behavior, reindeer, testosterone, vaccination

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Puerperal metritis in dairy cows: risk factors, efficacy of ceftiofur therapy and reproductive efficiency

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The objectives of this study were to assess the risk factors for puerperal metritis (MET), the effects of MET on milk yield (MY) and reproductive efficiency, and the efficacy of ceftiofur (CEF) therapy in Holstein dairy cows. The study was conducted in a commercial dairy herd (Cordoba, Argentina) where Holstein cows (N= 303) calving between April 15 and May 15, 2005 were enrolled.

Cows were body condition (BC) scored (1-5) and tail bled on -14 and 7 d relative to parturition. Rectal temperature (RT) was recorded on 5-7 days postpartum (dpp). Vaginal mucus (VM) was obtained with a gloved hand on 5-7, 21, 31, and 41 dpp, and was classified as VM0 (normal clear mucus), VM1 (clear mucus with pus flecks), VM2 (mucopurulent not fetid mucus), and VM3 (watery, purulent or brown-colored, fetid). Cows having a VM3 and RT <39.1˚C were categorized as having clinical metritis (CM), and those having a VM3 and RT ≥39.1˚C on 5-7 dpp were categorized as having puerperal metritis (PM). Clinical metritis and PM cows were randomly assigned to control (PLA) or CEF group (2.2 mg/kg for 3d; Ceobiotic®, Tecnofarm SRL, Argentina). Cure rate was assessed on 21 dpp by VM inspection as mentioned above. Cows having VM other than VM0 between 21 and 41 dpp were diagnosed as having CE. Plasma blood samples were analyzed for non-esterified fatty acids (NEFA), beta-hydroxy butyrate (BHB) and blood urea nitrogen (BUN) using commercial kits and IGF-1, insulin, and leptin by radioimmunoassay. Data were analyzed with PROC MIXED, PROC GENMOD and PROC PHREG from SAS®.

The risk for MET increased with abnormal calving (AOR [adjusted odds ratio] =2.58, *P*=0.008), as prepartum NEFA and BHB increased (AOR=1.001, *P*=0.177, and AOR=1.001, *P*=0.042, respectively). Conversely, risk of MET decreased as prepartum IGF-1 and postpartum BCS increased (AOR=0.652, *P*=0.144; AOR=0.054, *P*=0.092). The CM and PM cows had lower MY by 90 dpp than the non-MET cows (2235.62±172.11 vs. 2367.20±77.45 vs. 2646.56±82.10 kg, *P*=0.009; respectively). Puerperal metritis cows had lower risk for pregnancy rate by 100 dpp (AOR=0.19, *P*=0.001), higher risk for non-pregnancy rate by 200 dpp (AOR=1.93, *P*=0.088), higher risk for reproductive culling (AOR=4.12, *P*=0.062), lower hazard rates for pregnancy by 150 dpp than non-MET or CM cows (HR=0.753, *P*=0.004), and took longer to get pregnant than herdmates (129±3 vs. 111±8 vs. 109±3 days, for PM, CM and non-MET cows, respectively, *P*=0.001). Ceftiofur had no effect on cure rate 21 dpp (*P*=0.468), but reduced the risk for reproductive cull (AOR=0.134, *P*=0.038). In conclusion, the risk for MET increases with abnormal calvings and as NEFA and BHB increases, while the risk decreases as IGF-1 and BCS increase. Puerperal metritis has detrimental effects on MY and on reproductive efficiency since PM cows take longer to get pregnant and are at higher risk for culling. Lastly, CEF has no effect on cure rate but reduces the culling rate.

Keywords: Dairy cow, puerperal metritis, reproductive efficiency, risk factors

Clinical endometritis in dairy cows: risk factors and reproductive efficiency

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The objectives of this study were to assess the risk for clinical endometritis (CE), and the effects of CE on milk yield (MY) and reproductive efficiency in Holstein dairy cows. The study was conducted in a commercial dairy herd (Cordoba, Argentina) where Holstein cows (N= 303) calving between April 15 and May 15, 2005 were enrolled. Calving history and MY were obtained from dairy records. Cows were body condition scored (BCS, 1-5) and tail bled on -14 and 7 days (d) relative to parturition. Vaginal mucus (VM) was obtained with a gloved hand and observed by direct inspection on 5-7, 21, 31, and 41 days post partum (dpp), and classified as: VM0 (normal clear fluid), VM1 (clear fluid with pus flecks), VM2 (mucopurulent not fetid fluid), and VM3 (watery, purulent or brown-colored, and fetid). Cows having a VM3 on 5-7 dpp were categorized as having puerperal metritis (PM). Cows having VM other than VM0 between 21 and 41 dpp were diagnosed as having CE. Plasma blood samples were analyzed for non-esterified fatty acids (NEFA), beta-hydroxy butyrate (BHB) and blood urea nitrogen (BUN) using commercial kits and IGF-1, insulin, and leptin by radioimmunoassay. Data were analyzed with PROC MIXED, PROC GENMOD and PROC PHREG from SAS®. Abnormal calving increased the risk for CE (AOR [adjusted odds ratio] =2.21, P=0.019), and PM increased the risk for CE (AOR=2.21, P=0.032). Prepartum NEFA increased the risk for CE (AOR=1.003, P=0.045) while prepartum BUN reduced the risk for CE (AOR=0.853, P=0.147). Lastly postpartum BHB increased the risk for CE (AOR=1.001, P=0.10). Cows with CE had a trend for higher MY than non-CE herdmates (26.79±1.07 vs. 24.98±0.56 l/d, P=0.074). Cows with CE had lower risk for pregnancy rate by 100 dpp than non-CE herdmates (AOR=0.10, P=0.002), higher odds for non-pregnancy rate by 200 dpp than non-CE cows (AOR=2.87, P=0.011), and higher risk for reproductive culling (AOR=24.29, P<0.001). Also, CE reduced the hazard for pregnancy by 150 dpp (HR=0.30, P<0.001) and increased the calving to conception interval by ~30 d (mean±SE, 109±3 to 142±3, non-CE vs. CE cows, P<0.001). In conclusion, the risk for CE is increased in cows with abnormal calvings and PM, and it is also increased as prepartum NEFA and postpartum BHB concentrations are higher. Lastly, CE has detrimental effects on MY and on reproductive efficiency since CE cows take longer to get pregnant and are at higher risk for culling.

Keywords: Dairy cow, clinical endometritis, reproductive efficiency, risk factors

Late gestation hematology and serum biochemistry ranges in the canine fetus

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Introduction

Organ systems mature at different rates prenatally and postnatally varying by species. Normal hematology and serum biochemistry values can be evaluated based upon known ranges for a species, and can be used to determine the maturity of an organ system. The aim of this study was to document hematologic and biochemical parameters just prior to parturition, 61±1 days after the luteinizing hormone (LH) surge (term=65 days), which is clinically relevant when managing the timing of elective cesarean sections. The hypothesis was that late gestation (prenatal) blood values would not be different from those reported for puppies 1-3 days old (postnatal). In addition, in other species serum cortisol concentrations increase prior to parturition, indicating fetal readiness for birth. We also hypothesized that prenatal cortisol concentrations would fall within the adult normal range.

Materials and methods

Pregnant beagles (n=4) were ovariohysterectomized on 61±1 days after the LH surge. Cardiac blood samples were collected from fetuses (n=12) for serum chemistry, cortisol and complete blood count. Student's t-tests were used to compare prenatal and postnatal values using EpiCalc2000 (FSF, Inc., Boston, MA, USA). Significance was defined as $p<0.05$.

Results

Mean prenatal cortisol concentrations were outside of the normal range reported for adult beagles (0.61 to 4.75 ug/dL).[1] Many prenatal values were significantly different from postnatal values (Table).[2,3]

Conclusion

This is the first report of serum cortisol concentrations in late gestation canine fetuses. We speculate that the decreased biochemical indices measured prenatally were most likely due to organ prematurity, suggesting that in dogs significant organ maturation occurs in the last four days of gestation and first three days following birth. Further research is needed to elucidate organ maturation in late gestation canine fetuses.

Keywords: Canine, cortisol, fetal, hematology, serum biochemistry

Table	Prenatal Mean±SD	Postnatal Mean±SD
RBC count (x10^6/uL)	4.62±0.58	4.97±0.44[a]
Hemoglobin (g/dL)	13.57±1.67	14.0±1.4[a]
Packed cell volume (%)	44.92±5.70	43.1±4.0[a]
nRBC/ 100 WBC	16.67±23.28	12±8[a]
ALT (U/L)	9.17±3.56*	43.3±16.8[b]
Total bilirubin (mg/dL)	0.18±0.04	0.18±0.05[b]
Cholesterol (mg/dL)	105.42±30.11*	161±52[b]
Total protein (g/dL)	3.19±0.32*	4.26±0.35[b]
Albumin (g/dL)	2.27±0.24	2.29±0.26[b]
Total calcium (mg/dL)	13.07±0.74*	3.18±0.17[b]
Phosphorus (mg/dL)	12.06±0.89*	2.49±0.54[b]
Glucose (mg/dL)	213±70.20*	130±18[b]
BUN (mg/dL)	18.75±2.34*	72.9±24.9[b]
Creatinine (mg/dL)	0.64±0.26*	0.44±0.05[b]
ALP (U/L)	476.17±129.70 *	1106±757[b]
Cortisol (ug/dL)	5.10±0.93	
*$p<0.05$	n=12	[a]n=51, [b]n=34

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Assessment of tissue functionality after xenografting of canine ovarian cortex

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Ovarian tissue freezing protocols have been developed in several species, including the bitch, in our laboratory. This study was designed to evaluate the *in vivo* functionality of canine ovarian cortex after slow freezing, by using a xenografting model. Since the time of ischemia/reperfusion is known as a critical step of transplantation, the use of erythropoietin as an angiogenic factor has been evaluated to optimize the transplantation procedure.

Frozen-thawed ovarian tissue from five bitches was grafted for one, eight, or 16 weeks into the muscle of the hind limb of 45 Fox Chase SCID® Beige mice (Charles River Co., L'Arbresles, France). One part of the mice received erythropoietin (EPO, Janssen-Cilag Co, Issy-Les-Moulineaux, France) injections during 3 days post-surgery. All the mice underwent vaginal irrigation during the graft time, and endometrial status was controlled after euthanasia. Assessment of the follicle classification, density, morphology, and the stromal density was carried out by histogical analysis, whereas vascularization of the graft was quantified by immunohistochemistry with anti-alpha-*sma* antibodies (Abcys S.A., Paris, France). Data were analyzed by a one-way analysis of variance and a Bonferroni adjustment method using R software.

One week after transplantation, a loss of 94.7 and 96.3% of the follicular density was already visible for the EPO treated and non-treated group respectively, when compared to thawed tissue. Furthermore, primordial follicle distribution was lower (34.8% ± 17.4 & 38.2% ± 10.6, $P<0.05$) when compared to fresh tissue (83.7% ± 3.1) indicating that early follicles were very affected by this massive follicular loss, even if no significant differences was observed when considering the *sma* immunostaining before and after one week transplantation. Nevertheless, eight weeks post-graft, secondary follicles were still found (78.8% ± 7.0 & 85.4% ± 8.5 of the follicle population), and stromal density was significantly higher in EPO treated tissue compared to the 'one week' group. Even if fibrosis was noticed on several graft tissue, stromal cells tend to reorganize from eight weeks to reach a normal density at 16 weeks post-transplantation (52.9 ± 4.6 & 53.9 ± 6.2 stromal cells/0.01 mm²), when compared to fresh tissue (74.6 ± 5.7 stromal cells/0.01 mm²). Intact secondary follicles with more than 3 granulosa cell layers were also observed 16 weeks post-transplantation. Long term xenografting allowed follicular growth in canine frozen thawed ovarian fragments, even if massive loss occurred during the first week. The EPO treatment did not show a significant effect on vascularization but seems to improve the graft resumption.

Keywords: Xenografting, freezing, EPO, follicular growth, stromal cells

A single administration of a GnRH antagonist inhibited canine gonadal axis functionality for 14 days

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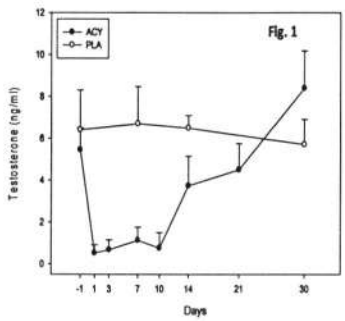
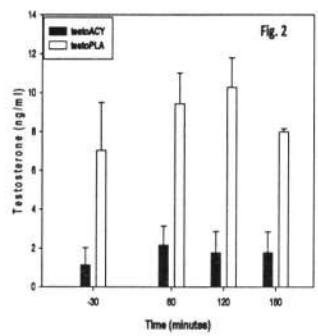
Gonadotropin releasing hormone (GnRH) antagonists, which bind to gonadotrope GnRH receptors and compete successfully with endogenous GnRH for specific receptors, have a great potential as contraceptives.[1] In male dogs, a single administration of the potent, third generation GnRH antagonist acyline, reversibly and safely decreased serum gonadotrophins and testosterone (T) concentrations for 10 days.[2] The functionality of the gonadal axis during antagonist treatment has not been described in this species. The objective of this study was to describe T response to GnRH challenge in GnRH antagonist-treated dogs over a 30-day period.

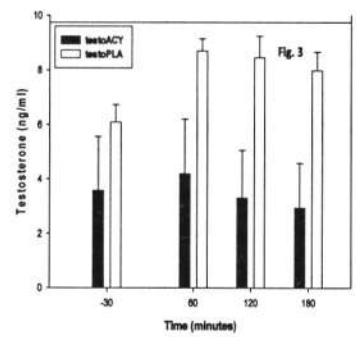
Eight reproductively normal mixed-breed dogs were randomly assigned to acyline (NIH, Bethesda, MD) 330 µg/kg sc (ACY; n=4) or a placebo group (PLA; n=4; day 0), and challenged with the GnRH agonist buserelin (Receptal®, Intervet, Bs As, Argentina) 0.2 µg/kg sc on days -7, 1, 3, 7, 14, 21 and 30. Blood samples for T determinations were collected before (-30 minutes) and 60, 120 and 180 min after the agonist injection. Serum T was measured by electrochemiluminiscense (Elecsys®, Cobas, West Sussex, England) and statistically analyzed by ANOVA for repeated measures (SPSS® Inc. Chicago, IL).

Before treatment (day -1) there were no differences in T serum concentrations between groups ($P > 0.1$). After the initial treatment, basal (-30 minutes) T differed throughout the days of the experiment between groups ($P=0.05$), varying in the ACY ($P < 0.01$) but not in the PLA group ($P > 0.1$; Figure 1). Furthermore, d 30 differed from days 1, 3, 7 and 10 in the ACY group ($P < 0.01$; Figure 1). On d -1, the stimulation tests had only a time effect ($P = 0.05$), although on d 7 ($P < 0.01$; Figure 2) and 14 ($P < 0.05$; Figure 3) the response differed between groups.

It is concluded that a single administration of the GnRH antagonist prevented canine gonadal axis from physiologically responding to agonistic challenge for 14 days. These results warrant further work on new GnRH antagonists in male dog reproduction.







Keywords: Canine, dog, GnRH antagonist, acyline, GnRH challenge

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Oral administration of an anti-inflammatory drug does not compromise the efficacy of intratesticular injection of zinc gluconate as a contraceptive for dogs

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Introduction

We have demonstrated the potential of a single intratesticular injection of a zinc gluconate solution (Testoblock; Biorelease Technologies, LLC, Birmingham, AL) as an irreversible contraceptive for male dogs. The results reported in a six-month study revealed that dogs were azoospermic 60 days after injection and histological and ultrastructural changes suggested irreversibility.[1] Recently, we observed that some dogs presented discomfort after the procedure and others[2] reported the same situation after using a similar drug in male dogs. In this study, an oral analgesic with anti-inflammatory action was administered for two days. One of the mechanisms of action of zinc when injected into the testis is the disruption of the Sertoli cell barrier and induction of a local immune and inflammatory response that causes cellular alteration and interruption of spermatogenesis.[1] Therefore, the use of an anti-inflammatory drug could affect this mechanism of action of zinc gluconate and compromise the efficacy of the chemical sterilization procedure.

Aim

The aim of this study was to examine whether the efficacy of zinc gluconate as a chemical sterilant would be compromised in the presence of an antiflammatory/analgesic agent, sodium dipyrone.

Methods

Ten sexually-mature mongrel dogs were assigned to 2 groups, a control group (n=5) and a treated group (n=5), and into each testis a single injection of zinc gluconate solution was administered. The treated group received sodium dipyrone (25mg/kg, TID, PO) that was administered after the procedure for two days. Zinc gluconate solution was injected at six different doses (0.2 to 1.0mL) based on testicular width (10 to 27mm). General attitude, ability to walk, scrotal alteration (pain, swelling, dermatitis), rectal temperature, semen analysis, hematology, and renal and hepatic function were performed on day 0 after injection and every two months for one year.

Results and discussion

No biting/licking was recorded after the injection in all animals. Transient testicular swelling was reported in both control and treated animals during the first three days after injection. Dogs that vocalized or did not want to eat after the procedure received dipyrone for two days and normal behavior was observed following treatment. At 60 days post-injection, all animals were azoospermic and this condition was observed until the end of experiment. There were no significant differences between treated and control groups for the clinical parameters evaluated and values for the parameters were within normal ranges for domestic dogs. Sodium dipyrone is a well-known non-steroidal anti-inflammatory drug used for acute and chronic pain. Beyond its analgesic importance dipyrone has anti-inflammatory properties that are only secondary to its main action. Therefore, this secondary function may not have affected the mechanism of action of zinc gluconate.

Conclusion

The administration of an analgesic drug (sodium dipyrone) after chemical castration of dogs using an intratesticular injection of a zinc-based solution does not compromise the efficacy of the procedure and contributes to animal welfare.

Keywords: Chemical castration, dog, sodium dipyrone, testis, zinc

Acknowledgments

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Accurate prediction of the timing for parturition and of the kittens' birth weight

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The two-fold goal of this prospective study was to predict both the parturition time using ultrasonographic measurements of the femur compared to the biparietal diameter and the kittens' birth weight. For this purpose, predictions were estimated using linear mixed-effects models on R software. This study was performed in 24 purebred queens with normal pregnancy. Cats were scanned from 35 days before parturition to the day of term, using a micro-convex probe. The best linear regression of the parturition time was $y = 37.864 - 0.193 \times x_1 + 1.227 \times x_2 - 0.615 \times x_3 - 0.832 \times x_4$. The variables were the femur length (x_1), the weight of the queen before pregnancy (x_2), the litter size (x_3) and the age of the queen (x_4). The 70% prediction level was $y \pm 1.6$ days. The kitten's birth weight was correlated to the calculated femur length at birth (x_6) and the wither height (x_5). The estimated weight (w) was determined using: $\log(w) = 0.692 + 0.011 \times x_5 + 0.005 \times x_6$. The best predicted level was obtained using femur length as compared to biparietal diameter. The duration of the gestation was increased with the weight of the queen before mating ($P<0.01$). The onset of the parturition was sooner when the femur was longer, and when the queen was older ($P<0.01$). The prediction of kittens' weight at birth would require a better accuracy of the pregnancy duration.

Keywords: Feline, ultrasound, parturition, fetal morphometry

Effect of semen type; inseminate volume, and sperm numbers on post-breeding inflammation in mares

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The duration and severity of post-breeding inflammation due to the presence of spermatozoa is a critical factor in the ability of the mare to become and remain pregnant. We hypothesized that uterine inflammation of mares inseminated with frozen semen (FS) would be less severe than mares bred with fresh cooled semen (CS), increasing the pregnancy rate. The objective of this study was to assess the inflammatory reaction clinically by cytology and biochemical components of uterine flushes of mares bred with CS or FS and determine effects on pregnancy rates.

Eighty-two mares were bred by artificial insemination (AI) with CS (n=44) or FS (n=38) on 104 cycles over a three month period. Mares inseminated with CS were bred within 24 hrs pre-ovulation with 500 to 1000 x 10^6 spermatozoa in volumes ranging between 50 and 100 mL deposited in the uterine body. Mares inseminated with FS were bred within one to four hrs post-ovulation with 50 to 100 x 10^6 spermatozoa in volumes ranging between 0.5 and 1 mL deposited at the tip of the uterine horn on the side of ovulation. Transrectal ultrasound examination and uterine lavage were performed on average 6.6 hrs post-breeding. Depth of fluid (cm) (UF) in the uterine body and degree of uterine edema post-insemination (0-5) (UE) were noted. The first 50 mL of recovered lavage fluid was evaluated for opacity (grade 0 = clear to 5 = mucopurulent) (FO), presence and number of PMNs (%), and protein level (mg/dL) (TP). Slides for cytological evaluation were prepared by cytocentrifugation (Cytospin4, ThermoShandon, Pittsburgh, PA) using disposal plastic chambers (Cytofunnel, ThermoShandon) and glass slides (Cytoslide, ThermoShandon). Total protein in lavage fluid was determined using the Dimension® clinical chemistry system. Statistical analysis was performed using a binary logistic regression model and chi square test to compare the effect of semen type on uterine inflammation parameters (mean ± SEM) and effect of inflammation on pregnancy rate at 15 days post-ovulation.

Pregnancy rate for mares inseminated with CS or FS were not statistically different (61.5% vs 63.3%). There was no significant difference between the two insemination methods with regard to UF post-AI (6.37 ± 0.57 vs 3.95 ± 0.49); FO (2.32 ± 0.16 vs 2.36 ± 0.19); UE post-AI (2.26 ± 0.15 vs 1.90 ± 0.13); PMNs (%) (1.26 ± 0.18 vs 1.27 ± 0.21); and TP (5.13 ± 1.49 vs 26.27 ± 9.8). Non-pregnant mares results were UF post-AI (6.01 ± 0.56 vs 5.95 ± 0.46); FO (2.76 ± 0.18 vs 2.15 ± 0.14); UE post-AI (2.55 ± 0.17 vs 2.27 ± 0.17); PMNs (%) (1.38 ± 0.20 vs 1.50 ± 0.23); and TP (9.85 ± 3.68 vs 7.33 ± 1.82). No significant differences were observed between pregnant and non-pregnant mares. These results provide preliminary information suggesting that PR is independent of UF collection, FO, UE, PMNs and TP content as long as flushing is performed in timely manner following breeding and ovulation.

Keywords: Endometritis, uterine cytology, mare, semen preservation, seminal plasma

Evaluation of a modification of the McKinnon technique to correct urine pooling in mares

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Abstract:

The objective of this trial was to determine whether the incidence of defects in mucosal tunnels created to correct urovagina in mares could be decreased by modifying the commonly used McKinnon technique of urethroplasty. The urethral fold of 30 mares, none of which suffered from urovagina, was split transversely into dorsal and ventral shelves, and the ventral shelf was used to help create a urethral extension. The dorsal shelf was stretched caudally and sutured to the extension so that it covered at least the cranial half of the extension. For 20 mares, a relaxing, vaginal incision was created cranial to the external urethral orifice to enable the dorsal shelf to be retracted further caudally. Ten mares developed a defect in the extension, but none developed a defect in that portion covered by the dorsal shelf of the urethral fold. Four of the 10 mares that did not receive the relief incision and six of 20 mares that did receive the relief incision developed a defect in the extension. Several defects were so small they could be detected only by inserting a dye, under pressure, into the tunnel. Modifying the McKinnon technique of urethral extension by transversely splitting the urethral fold and retracting the dorsal half may help prevent a defect from forming at least in the cranial portion of the extension. The dorsal shelf can be retracted further caudally by creating a relief incision on the floor of the vagina.

Keywords: Urethral extension, urovagina, urine pooling, mares

Comparison between centrifugation and filtration process to concentrate stallion sperm before cooling

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Centrifugation of stallion semen is a necessary step for the stallion semen cryopreservation procedure, allowing the concentration of sperm. Centrifugation is also required for maximizing spermatozoa quality in semen from some particular stallions subjected to cooled transport because of the detrimental effects of seminal plasma. However, the centrifugation process has potential deleterious effects, including reduction in sperm quality as well as loss of sperm numbers. A recent publication from our laboratory has shown the efficiency of a filter made of a hydrophilic syntetic membrane to concetrate stallion sperm. The present experiment aimed to verify the effect of this novel system on cooled stallion semen quality. One ejaculate from a total of 30 stallions was collected. After collection the ejaculate was split into three aliquots and submitted to the following procedures before cooling at 15°C for 24 hours: G1—diluted with skim milk extender, G2—fresh semen filtration, and G3—centrifugation (500*g* for 10 minutes). G2 and G3 samples were resuspended with the same milk based extender used in G1. Motility parameters were analyzed by CASA and plasma membrane integrity by fluorescent probes. No differences ($p>0.05$) were observed 24 hours after cooling on total motility (TM; 37%, 51 %, and 47%), progressive motility (14%, 21%, and 17 %), membrane integrity (35%, 45%, and 47%), respectivelly for G1, G2 and G3 when samples from all stallions were evaluated. However, when data only from bad cooler stallions (TM <30% after cooling) were evaluated, a significant improvement ($p <0.05$) in TM was observed in G2 and G3 groups (13 %, 36 %, and 33%, respectively for G1, G2 and G3). We conclude that the filtration technique was practical, fast and safe for concentration of stallion sperm before cooling and that removal of seminal plasm is beneficial to some particular stallions.

Keywords: Stallion, semen, seminal plasma, filter, cooling

Mycobacterium intracellulare isolation from equine fetal membranes

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Mycobacterium sp. is classified as a zoonotic pathogen; the most recognizable of which are tuberculosis and leprosy. The most documented equine cases of *Mycobacterium sp.* infections present as granulomatous enteritis and colitis.[1] The non-tuberculosis forms of *Mycobacterium avium* complex (MAC) result from a slow-growing bacillus that is ubiquitous in the environment. The postulated route of transmission of MAC from animal sources to humans is by inhalation, ingestion or the percutaneous route.[2] MAC has been implicated as a cause of equine abortion; however, subspeciation was not performed in those cases.[3,4]

A pluriparous 15 year old Anglo-Arab mare foaled a full-term (344 days) filly on October 6, 2010, without assistance. Gross examination of the membranes revealed a weight of 3.6 kg, with a brown, viscous plaque adherent in a five cm diameter area of the uterine body. Differential diagnosis included a Nocardia-type placentitis, which frequently manifests as a thickened uterine body region overlain with mucoid exudate. Ziehl-Neelsen staining of a direct smear of the lesion revealed a considerable number of gram positive, acid-fast bacilli. A sample was submitted for analysis to the Queensland Mycobacterium Reference Laboratory. The Mycobacterial species determination was performed using a 16S sequencing procedure, leading to identification of *M. intracellulare*.

This case of *M. intracellulare* reiterates the necessity of biosecurity procedures when handling fetal membranes. The novelty regarding the diagnosis of *Mycobacterium intracellulare* placentitisis that infection has not previously been reported after delivery of a viable foal.

Keywords: *Mycobacterium intracellulare*, equine, fetal membranes, placentitis, MAC

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Renal cystadenocarcinoma arising from ovarian adenocarcinoma in a three year old Doberman bitch

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Primary ovarian tumors are uncommon in domestic animals, while cystadenocarcinoma is the commonest ovarian epithelial tumor in dogs. Renal cystadenocarcinomas have been reported in German Shepherd Dogs and Golden Retrievers, however, a renal cystadenocarcinoma associated with an ovarian cystadenocarcinoma has not been previously described in the literature. A three- year- old Doberman was presented with complaint of lethargy and weightloss. Physical examination revealed a mass in the right abdomen. Laboratory abnormalities included anemia, azotemia and metabolic acidosis. The mass was not opacified on excretory urography but the right renal pelvis and ureter were distended. The right calyces, pelvis and proximal ureter were dilated while the distal part of the right ureter was not observed. Transcutaneous abdominal ultrasound of the dog revealed an oval shaped mass located proximal to the bladder and distal to the caudal pole of the right kidney. The mass consisted of a central anechoic area surrounded by hypoechoic tissue. Following laparotomy, the mass was located in the right half of the abdominal cavity between the kidney and ovary. The mass was well-vascularized and attached firmly to the abdominal wall dorsally, while the right ovary was attached to the mass through a thin connective tissue band. Nephroureterectomy and ovariohysterectomy were performed and the resected tissues were submitted for histopathology. Histologically, the renal mass showed the papillary and tubular patterns of renal cystadenocarcinoma. The papillary projections and tubules were lined by pleomorphic, cuboidal to columnar epithelial cells. The cells were small, with somewhat basophilic, non-vacuolated cytoplasm. Their nuclei were round to oval and contained prominent single or double nucleoli. The mitotic rate was high. The tumor was highly vascularized and there was necrosis of tissue far from blood vessels. In addition, the ovary was normal in size and showed papillary pattern of mucinous cystadenocarcinoma. The bitch was euthanized owing to poor improvement. At necropsy of the left kidney revealed an end-stage kidney. It was concluded that the renal cystadenocarcinoma must have arisen secondary to a primary ovarian cystadenocarcinoma through stromal invasion.

Keywords: Renal cystadenocarcinoma, ovarian cystadenocarcinoma, Doberman, inbred, dog

Fixed-time AI pregnancy rate following insemination with frozen heterospermic semen

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This study aimed to evaluate the effects of conventional or heterospermic frozen semen on pregnancy rates in fixed-time artificial insemination (FTAI) protocols for post-partum Nelore cows with suckling calves. The trial was conducted on four farms at Mato Grosso do Sul in Brazil. We used 459 cows (50-110d post partum) with body score condition between 2.5 and 4.5 (scale of 1 to 5). Animals received estradiol benzoate (EB; 2.5 mg, i.m., Estrogin® Farmavet, Brazil) and an intravaginal device containing progesterone (1.9g progesterone, CIDR® Pfizer Animal Health, Brazil) in a random stage of the estrous cycle (D0). At D7 animals were treated with dinoprost trometamine (12.5 mg im; Lutalyse® Pfizer Animal Health). At D9 the CIDR® was removed and the animals were treated with eCG (300 IU, Novormon® Intervet/Schering-Plough Animal Health, Brazil), and estradiol cypionate (0.5 mg, i.m, ECP® Pfizer Animal Health). At D11 animals were inseminated with either: conventional semen from Nelore bull A (group 1, n=119), conventional semen from Nelore bull B (group 2, n=114), conventional semen from Nelore bull C (group 3, n=111), or with heterospermic semen from bull A + B + C in the same straw (group 4, n=115). Pregnancy was diagnosed by ultrasonography (Mindray DP 2200 vet) 32d after FTAI. Data were analyzed using Chi-Square test. There was no effect of farm on pregnancy rate ($P \geq 0.05$). Pregnancy rate of group 1, 2, 3 and 4 were 57.2%, 48.5%, 57.2%, and 56.9%, respectively ($P \geq 0.05$). We conclude that the use of frozen heterospermic semen did not improve the pregnancy rate of Nelore cows inseminated with a fixed-time AI protocol.

Keywords: Heterospermic semen, Nelore, fixed-time artificial insemination, pregnancy rate

Effect of unilateral orchidectomy on testicular characteristics of the domestic cat

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The aim of this study was to describe the effect of unilateral orchidectomy (UO) on testicular characteristics of mature domestic cats. Five, 1 to 2 y old, crossbreed male cats were unilaterally orchidectomized on d 0 (right testis) and d 60 (left testis). All the animals were exposed to $\geq$ 12 h of daylight for two months before and after the first hemicastration. After surgical removal, the testes were weighed and measured. Testicular volume and gonadosomatic index[1] were also calculated. The testes were fixed in Bouin's solution and stained with hematoxylin and eosin. In twenty rounds tubular profiles the maximum, minimum and medium tubular diameters; major and minor axes, area, perimeter and germinal epithelium height were measured (Image Pro Plus; MediaCybernetics, Bethesda, MD). The volumes of the different testicular tissue components were determined using an intersection grid on 40x photographs. For this, fifteen fields were chosen randomly and scored for each animal. Points were classified as spermatogonia, primary and secondary spermatocytes, round and elongated spermatids, spermatozoa, Leydig and Sertoli cells, intertubular compartment, basement membrane, lumen or cellular debris. The total length of seminiferous tubules was also obtained.[1] Both groups (d 0 vs. d 60) were compared by Student's t test and P values < 0.05 were considered significant. No significant differences between testes groups were found for any of the gross and microscopic parameters assessed (mean±SEM): testis weight (1.54±0.4 g vs.1.7±0.2 g), length (1.94±0.1 cm vs.1.92±0.8 cm) and width (1.04±0.1 cm vs.1.04±0.1 cm), volume (0.95±0.1cm^3 vs. 0.95±0.1 cm^3), gonadosomatic index (0.03±0.01 % vs. 0.04±0.01 %), maximum (240.5±29.8 µm vs. 250.8±18.6 µm), minimum (166.6±24.4 µm vs.194.1±13.1 µm) and medium (202.6±26.2 µm vs. 220.9±14.9 µm) tubular diameters, major (240.9±29.1 µm vs. 247.5±18.3 µm) and minor (171.8±24.7 µm vs. 200.4±12.6 µm) tubular axes, area (35356.2±8482.8 µm^2 vs. 39622.9±5193.4 µm^2) and tubular perimeter (668.1±84.8 µm vs. 718.7± 47.7µm), germinal epithelium height (58.6±7.5 µm vs. 55.3±5.3 µm), spermatogonias (0.056±0.1 cm3 vs. 0.052±0.1cm^3), primary spermatocytes (0.10±0.1 cm^3 vs. 0.11±0.1 cm^3), secondary spermatocytes (0.003±0.001 cm^3 vs. 0.002±0.01 cm^3), round spermatids (0.12±0.1 cm^3 vs. 0.13±0.01 cm^3), elongated spermatids (0.07±0.01 cm^3 vs. 0.066±0.01 cm^3), spermatozoa (0.04±0.01 cm^3 vs. 0.03±0.01 cm^3), Sertoli cells (0.064±0.01 cm^3 vs. 0.072±0.01 cm^3), Leydig cells (0.04±0.01 cm^3 vs. 0.04±0.01 cm^3), intertubular compartment (0.12±0.02 cm^3 vs. 0.12±0.02 cm^3), lumen (0.2±0.04 cm^3 vs. 0.3±0.03 cm^3), cellular debris (0.02±0.01 cm^3 vs. 0.01±0.0.1 cm^3), tubular- intertubular compartment proportion (7.17±1.2 vs. 7.29±1.3), basement membrane (0.02±0.01 cm^3 vs. 0.02±0.01 cm^3) and total tubular length (38.73±10.5 m vs. 32.66±6.2 m). To our knowledge, this is the first investigation to describe the effect of UO in domestic cats. According to these biometric and morphometric results, adult cats, similar to rodents,[2] do not develop compensatory hypertrophy after UO.

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