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

Conference Proceedings

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*
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- All pages are to be double-spaced
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- 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.
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- 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;68:817-30

  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; 54:757-69.

  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 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.
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<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 Society for Theriogenology

It is with great pride and enthusiasm that we present this year's proceedings for the SFT Annual Conference as the first issue of *Clinical Theriogenology*. This journal is the result of collaboration between members of the Society for Theriogenology and the American College of Theriogenologists and their collective desire to provide a venue through which clinically relevant scientific information related to the practice of theriogenology could be disseminated.

In the past this valuable material may have been exchanged among practitioners in more informal settings with limited opportunity to reach a broader audience, making it difficult for those outside these circles to grow in knowledge and proficiency in the practice of theriogenology. It is our desire that the growth of our specialty possess no such limitations and that the veterinarian interested in expanding their expertise in theriogenology find in this journal a valuable resource through which to obtain this knowledge.

We are very excited about this new territory and look forward to spending many years together.

Ana Adams
President, Society for Theriogenology
August 2009

From the President of the American College of Theriogenologists

It is a great pleasure to be asked to offer some remarks on the inaugural volume of *Clinical Theriogenology*. I am very excited about this opportunity for theriogenologists from around the world. Many have asked 'why a new journal?' and 'what are the chances for success of this journal?. These are legitimate questions and I will offer some brief answers in what follows.

I believe that the scope of *Clinical Theriogenology* will fill the need to provide authorative science-based reviews and clinical studies to theriogenologists and veterinarians with strong interest in the practice of theriogenology. I see this journal developing in the near future as the scientific forum for comparative clininal theriogenology. The second function of this publication, that we desperately need, is to serve as an official journal for our college.

As to chances of success, I believe that the wonderful editorial board in place under the leadership of Dr. Youngquist is the first catalyst for success. More importantly, I think that it is the duty of our membership to contribute to the success of our official journal by submitting original clinical papers as well as encouraging their trainees or veterinarians to submit papers within the scope of the journal. I particularly urge diplomates to encourage and help practitioners capitalize on the wealth of clinical observation they have accumulated from the field.

Ahmed Tibary
President, American College of Theriogenologists
August 2009

Proceedings of the Annual Conference of the Society for Theriogenology

August 25-29, 2009

Albuquerque, New Mexico, USA

Clinical Theriogenology

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Foreword

August 2009

Herewith for your approval, the inaugural issue of the journal *Clinical Theriogenology*, founded as the official journal of the Society for Theriogenology and the American College of Theriogenoloists. The Society's Board of Directors has carefully considered undertaking publication of a refereed professional journal for several years approved the concept at its January 2009 meeting. The Society and College are fortunate to have among their members talented academic clinicians and practitioners who are willing to serve on the editorial board and I look forward to working with them to develop *Clinical Theriogenology* into a useful educational instrument. The original purpose of the Society was to serve as a resource for dissemination of information and this venture is the next step in that mission.

In assembling this first issue which contains the proceedings of the Society's 2009 Annual Conference, it has been a pleasure to work with the section chairs and the authors who have contributed their time and talent to prepare the manuscripts.

As we look toward the future, please become an active participant in the educational process and consider submitting your clinically-oriented manuscripts for publication in *Clinical Theriogenology*. This journal belongs to every member of the Society and the College so please feel free to share your comments and suggestions with the editor or members of the editorial board.

R. S. Youngquist
University of Missouri
Columbia, MO

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2013
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2014
August 5-August 9
Portland, Oregon

The 2009 Bartlett Address

Theriogenology Experiences and Reflections

B.E. Seguin
Professor Emeritus, College of Veterinary Medicine, University of Minnesota, St Paul, MN, USA

Dr. Bartlett's Influence

To start this story I go back to my first year in veterinary school at the University of Minnesota. I worked in the Anatomy Department, and one of my duties was to make bone preparations for teaching and display purposes. One day I was asked if I would like to take on some extra work in this area and if so I should contact a Dr. Bartlett at American Breeders Service (ABS) in DeForest, WI. I did not know Dr. Bartlett but I certainly knew of the ABS Company, and was therefore very excited to find out what this might be about.

Now I'd like to go back several years before to my teenage years in my hometown of Alma Center in west-central Wisconsin. I was a "city-kid" if that is possible in a town of some 400 people. My dad introduced me to all kinds of animals but he especially liked birds and we raised chickens, ducks, pheasants and homing pigeons. I was more interested in dairy cattle. All six of the major breeds were raised on farms in our area. But Guernseys were my favorite as they were raised by my good friend Fred Moseley and on the Seguin family farm near Eau Claire, WI. As artificial insemination (AI) increased in popularity, I became intrigued with bull studs, semen technology and the work of AI technicians. So I was well aware of the ABS Company. The local veterinarian lived just a few houses away. I found his practice in general, but especially the reproductive part, compelling. So my intention for going to veterinary school was to work with cattle, and especially cattle reproduction in dairy practice or possibly the AI industry.

So I was very excited to find out what Dr. Bartlett had in mind. (Wouldn't a four-year scholarship be wonderful!)? As you can guess, what he was looking for was someone to provide pelvis preparations for use in their training programs for AI technicians. I was able to provide the specimens so my first paycheck for theriogenology work was thanks to Dr. Bartlett! But the real reward for me was the chance to get to know the veterinary staff at ABS: Drs. David Bartlett, Les Larson and Willis Parker. From that point on, the possibility of a career similar to theirs was my motivation whenever needed to get through veterinary school. A fact that is not well known is that Dr. Bartlett was the first head of the Ob/Gyn Department in the College of Veterinary Medicine at the University of Minnesota. In 1984 Dr. Bartlett gave the first of these addresses at this same meeting in Denver, Colorado. I encourage you to read that paper as it is a classic reflection of Dr. Bartlett's professionalism, intellect and dedication to our profession and specialty.[1] So I am deeply honored to speak to you today as this is the 25th anniversary of the first Bartlett address given of course by Dr. Bartlett himself.

Student at the University of Minnesota Veterinary College

As veterinary students at Minnesota in the 1960's most of us had heard the legend of Dr. Ray Zemjanis long before we were to have him as a teacher. He was on sabbatical for our 3rd year when we started our theriogenology courses. However we were not shortchanged, as a very capable group remained to carry on: Dick Schultz, Mel Fahning, Louis Archibald, Charles Gibson, Dennis Copeland, John Ellery and Bob Wescott. They were all superb teachers, strong mentors and excellent theriogenologists. During that year we took cow palpation lab, which included several early morning trips to the Bartusch slaughterhouse about 3 miles from campus. On these trips cows to be slaughtered that morning were palpated and findings recorded by each student. The reproductive tracts were recovered and returned to the Therio lab for review (and grading) that noon. This nearly instant feedback helped us master the art and science of cow palpation and reproductive physiology. This slaughterhouse resource was the backbone of the Minnesota program and where we really learned the techniques described in the Zemjanis textbook *Diagnostic and Therapeutic Techniques in Animal Reproduction*.[2] Ray Zemjanis returned as we started our 4th year. Those of you who knew him you can imagine the surprise we experienced that first day in lecture. Even though his reputation had proceeded him and we knew we could learn a great deal from this man, his hardcore, old school, no nonsense approach was a shock to say the least. If we answered when doing cow palpation with "I think I found ------", he would respond, "Your clients won't care about what you THINK you found, they expect you to KNOW what you found." Of course he was right and we gained great respect for the teaching system he established and the principles he professed.

Colorado State University

Toward spring of my 4th year, a notice was posted that the veterinary college at Colorado State University (CSU) was looking to fill an internship position in bovine reproduction. Anyone interested should contact Dr. Ed Carroll at the "Bull Farm". That address sounded perfect! I went to the library to see what I could learn about Dr. Carroll and found his paper on BSE of 10,940 beef bulls,[3] which was the basis for the formation of the group that

would become the Society For Theriogenology. I applied and was fortunate to be offered the position. The Bull Farm was an exciting place where I worked with Drs. Carroll, Les Ball and Lloyd Faulkner. We did lots of bull work and more cow work (both beef and dairy) than I had imagined. Also present at CSU were Dr. Bill Pickett with his equine research program and Dr. Jim Wiltbank working on beef cow reproductive management systems. Their detailed approach to controlled experimental design in animal reproductive studies made a strong impression. I had many "interesting" mornings with Dr. Pickett and his graduate students collecting semen in their seasonality study in stallions and in a boar project. These experiences plus a growing concern at that time about seminal vesiculitis in bulls started me thinking that research in bovine reproduction and possibly even graduate school might be something to consider. Dr. Carroll and I did a small trial on seminal vesiculitis in yearling bulls in the hopes of creating the basis for a possible graduate degree project. [Unfortunately Ed's life was far too short; he was a valuable resource to our profession.] The days at CSU flew by.

Michigan State University

Fortunately for me there was a bovine theriogenology residency/MS spot at Michigan State University with Drs. Dave Morrow, Wayne Oxender and Fayne Oberst. I got off to rocky start with Dr. Morrow when he wanted me to write an application for an NIH postdoctoral fellowship. My first reaction was to question if a veterinarian was even eligible, but indeed we are (or were). About the last thing I thought I wanted to be doing was to be confined to a desk when there were field trips, clinic cases and experiments to be done. But I did the application and have been grateful to Dr. Morrow ever since as it was funded about 2 years later. My Master's thesis was on the cycle-altering effects of endometrial irritation in cows,[4] which started my career-long search for better control of the timing of estrus and ovulation in cattle. Two review papers by Dr. O.J. Ginther were available on the utero-ovarian relationship in cattle.[5,6] I still feel these should be must read literature for graduate students and/or residents in theriogenology. At that time we were still talking about the uterine luteolysin and the luteolytic factor, but this would soon change.

The Dairy Science Department at MSU had a very active Reproductive Physiology group that included Drs. Harold Hafs, Alan Tucker, Ed Convey, Jack Britt and Wayne Oxender plus a large number of graduate students. It was the exciting place to be. I was accepted into a Ph.D. program with the Dairy Science group with Wayne Oxender as my advisor. Graduate students were officed six per room so there were many stimulating discussions. There were always lots of trials being planned and conducted and many needed the assistance of a veterinarian so my days were always busy.

If you know Wayne Oxender, you know that something interesting was always going on when he was around. In those days, Select Sires and Michigan Animal Breeders had a bull stud on the edge of campus. They allowed us to use 25 two-year-old Holstein bulls housed in tie stalls for our experiment. The purpose was to study five anterior pituitary hormones possibly involved in the process by which false mounting bulls increases the number of sperm per ejaculate. Each bull was treated and then bled via the tail vein eight times in a 24-hour period. That barn was all muscular energy, testosterone and flying hooves. Somehow we all survived and I was able to present the project at the ASAS meeting in Lincoln, NE.

Prostaglandin! Today the activity of prostaglandin F2α (PGF2α) in cattle is taken for granted but in 1972 prostaglandin was pretty much unheard of. One day at the weekly graduate seminar Tom Louis reviewed some recent work in lab animals that showed some negative effects on corpus luteum (CL) function by a compound, which was thought to be what we had been calling the uterine luteolysin. As I remember many of us present thought "This is a Dairy Science Department, what in the world does this work have to do with cows?" Little did we appreciate where this was going to take us. Shortly thereafter, Dr. Hafs went to visit Dr. Jim Lauderdale at the Upjohn Company about the possibility of some trials with a new compound that was PGF2α. Soon Tom Louis, John Stellflug, and I were sent to the University's dairy to select heifers and cows for the initial trials with this product. Late one night we were in the research barn getting everything ready for the start of the project when the manager of the dairy farm walked through. It must have been obvious to him that we could hardly contain our excitement. He asked what company was sponsoring the project. We were only thinking about how the project would get us started on our graduate work but he was looking for an investment opportunity! In the first trials cows were treated by intrauterine infusion in the ipsilateral vs. contralateral horn to the CL and the heifers by intravaginal deposit vs. IM injection in diestrus vs. metestrus. There were no adverse reactions and to our amazement, the CLs that were present at the time of treatment were soon disappearing. When the heifers went out for heat detection on the third day after treatment they lined up in standing heat like elephants in the circus. These two projects resulted in two of the first publications on the action of PGF2α in cattle.[7,8] Many other experiments with PGF2a followed to further define dose effects, routes of administration, intervals to estrus and ovulation, etc. Then it was time for fertility trials where we had very good results.[9] A review of potential uses of PGF2α in cattle was presented at the first SFT conference by Wayne Oxender.[10]

There were some other PGF2α experiments and experiences with unexpected events. Several surprises occurred at the Chatham Experiment Station in the Upper Peninsula of Michigan where we used beef cattle for one of the first PGF2α fertility trials. The first was that we would take a commercial plane to Chatham, which sounded great, but it was a very bumpy ride in a small commuter plane and it made 3 or 4 stops! A second surprise was the weather. We left Lansing on a beautiful spring day but in the UP it was still winter with more than a foot of snow still on the ground. Another surprise was the cattle. Some of you will remember the Chianina beef breed that was imported from Italy for crossbreeding to add some size to our native beef cows. It turned out their other highly heritable traits were how high they could jump and how slow they were to reach puberty. The heifers used were ½ and ¾ Chianina yearlings. Although well grown and in good condition, many were not yet cycling and they all could jump. It was a long day. You have probably seen the warning on the Lutalyse® bottle that says exposure to this product may cause breathing problems or an asthmatic attack in some people. I have never seen such a problem with the commercial product but for these first trials PGF2α came to us in powder form that we had to dissolve in PSS. I had usually done this in a large well-ventilated room but that day I did it in a closed up car. As I mixed the two, some of the PGF2α powder became airborne and I experienced a weird respiratory episode. This did not last long but I was convinced of two things: PGF2α is a potent substance and it is true that it's half is very short. On our last trip to Chatham an experiment station cowboy named Blacky took us Coho fishing in a local river. When we could not get a bite by hook and line, he just illegally used a dip net to catch a few under a state highway bridge.

There was also some mare reproductive research done in our group. Pat Noden was another of the graduate students. Her Ph.D. thesis was on using PGF2α for estrus control in mares.[11] The day the first mare was treated I was at the campus dairy barn. I received a phone call that there was an emergency at the Bennett farm where the research horses were kept. By the time I was contacted and got over there, all was quiet and no one was around. The only thing that seemed unusual was a large wet spot in the treatment area! While we had never seen any side effects from PGF2α in cattle, Pat had just seen the first sweating reaction in a mare caused by PGF2α treatment. This reaction in mares to PGF2α is well known now but was a shock that day.

Another surprise occurred when Dr. Hafs wanted to try PGF2α in bulls. He had a research interest in sperm output and collection efficiency of AI bulls. He wanted to see if an injection of PGF2α might mimic the affect seen with false mounting. The initial trials were done in rabbits where the most obvious affect was a marked increase in fecal expulsion.

One disappointing aspect of the early PGF2α work for me personally was that we did not have approval to do research trials in lactating dairy cows. This was my major interest area and where most of us thought the greatest use would occur.

Gonadotropin Releasing Hormone: At the same time that the PGF2α research was starting at MSU, Drs. Jack Britt and Ed Convey and several graduate students were studying uses of gonadotropin-releasing hormone (GnRH) in cattle reproduction. An early project by Britt et al. described the basic effect of GnRH on LH release and showed great promise for the use of GnRH to induce early postpartum ovulation and CL formation in cows.[12] Another project that became my Ph.D. thesis compared the luteotropic effects of hCG and GnRH in normal cattle and in dairy cows treated for ovarian follicular cysts.[13,14] An interesting part of this work happened when several bovine practitioners were asked to find cows with follicular cysts for this trial. Even though it was specified that the cysts had to be follicular in type, 40% had enough progesterone to be luteal cysts if not normal CLs. When in 10 to 14 days I went to examine and treat the cows, most of that 40% no longer had the ovarian structure originally thought to be a follicular cyst. A review of potential uses of GnRH in cattle was presented at the first SFT conference.[15]

University of Minnesota Faculty

With completion of my graduate program at MSU, it was time to find a real job. Back at the University of Minnesota there was a temporary position to be filled while Ray Zemjanis went to Africa to teach for two years. The Theriogenology group had changed greatly since my graduation. Now Ed Mather, Howard Whitmore, Borje Gustafsson (newly hired from Sweden) and Bob Wescott were the faculty, and residents included Shirley Johnston, John Hurtgen and Rolf Larsen. Also working closely with the Theriogenology group were the Reproductive Physiology group from the Department of Animal Science:
Ed Graham, Bo Crabo, Alan Hunter and Jon Wheaton. Members of both departments participated in the Theriogenology Graduate Program, in which 12 to 15 veterinarians were enrolled. Later Ed Mather moved to Michigan State University and Borje Gustafsson and Howard Whitmore went to the University of Illinois. They were replaced by Ray Zemjanis returning from Africa, Norm Williamson from the University of Melbourne and myself. After completing her Ph.D., Shirley Johnston joined the faculty as Small Animal Theriogenologist. Dr. Mel Fahning returned to the program after several years in private practice and bovine ET business. These were

rewarding times with an excellent teaching and clinic program for veterinary students, and productive teaching and research with veterinary residents and graduate students in Theriogenology.

With a series of graduate students we continued studying applications for PGF2α and GnRH in cattle. Now we had two PGF2α products (Estrumate® and Lutalyse®) to work with. Despite ads to the contrary we could find no significant difference in the luteolytic potency between these two products in dairy cows.[16] We did however make some interesting observations on factors affecting results with either product when treating unobserved estrus in dairy cows. We did several fertility trials in cooperating private beef herds. Cooperating farmers would assure us that the entire herd was ready for the breeding season when we planned for the first PGF2α injection. But a pretrial palpation would almost always find at least a few pregnant cows! A good lesson for everyone involved was that, at least in the first year of a PGF2α controlled breeding program for beef herds, never treat cows prior to checking that some might still be pregnant. We also looked at the efficacy of the PGF2α products to induce abortion in feedlot heifers[17] and causes of the variation in the timing of estrus after PGF2α treatment. Then the fun began when it became possible to do PGF2α application trials in lactating dairy cows; first in individual cows with unobserved estrus[18] and later in herd breeding programs.[19,20] Today many dairy herds use multi-product, multi-treatment programs for breeding that eliminate estrus detection altogether.

One of the real highlights of my faculty career came in 1984-85 when I was able to go on sabbatical leave at the Swedish Veterinary College. At that time the Ob/Gyn Department included Drs. Stig Einarsson, Kjell Larsson, Hans Kindahl as well as many excellent staff and graduate students. Their courses, facilities, clinical service and research were first class. I could not speak Swedish so I did very limited lecturing; rather I mostly helped the graduate students with their research projects. My own research efforts that year were to focus on measuring a PGF2α metabolite. This was thwarted by a small technical detail that became a painful lesson. We changed to a new source of tubes for blood collection without testing to see if the new tubes affected assay results. After all samples had been collected, we learned that the new tubes somehow interfered with the assay negating nearly a years' work. My family and I will never forget the hospitality and friendships we enjoyed that year.

We returned to Minnesota to some changes. Ray Zemjanis retired and was replaced by Harry Momont. Norm Williamson moved to the veterinary school in New Zealand and Jerry Olson joined us. Shirley Johnston moved into administration. Harry Momont moved on to the veterinary school in Wisconsin. Margaret Root Kustritz came on board to work in small animal theriogenology and Mats Troedsson joined our group specializing in equine theriogenology.

One Last Trial

Over the years we had done many projects looking at various means to maximize control of the timing of estrus and ovulation in cattle. These included endometrial irritation, estradiol cypionate (ECP), PGF2α, GnRH, hCG, removal of the ovary containing the preovulatory follicle,[21] etc.

The last experiments I undertook involved two protocols that were tested for potential to improve AI submission and pregnancy rates achieved by using PGF2α (Lutalyse®, Pfizer Animal Health, New York, NY, USA) in dairy cows with unobserved estrus judged to have a PGF-responsive CL by ovarian palpation. In Expt 1, 151 cows were selected and treated with PGF2α. Cows in the experimental group were treated with 1.5 cc (3 mg) of ECP IM at 30-34 hrs after PGF2α while controls had no further treatment. Significantly more cows treated with PGF2α plus ECP were seen in estrus and AI'd within 6 days of PGF2α than were cows treated only with PGF2α (91% vs. 53%). Estrus behavior was more tightly synchronized for cows treated with PGF2α plus ECP than for cows treated only with PGF2α. Resulting conception rates were similar (55% vs. 58%). Therefore pregnancy rate 6 days after PGF2α was higher for cows treated with PGF2α plus ECP than for cows treated only with PGF2α (50% vs. 31%). In Expt 2, 197 cows were similarly selected and treated but all cows were AI'd at 72-76 hrs after PGF2α without regard for estrus behavior. There was no significant difference in pregnancy rates between groups, 40% for 98 PGF2α plus ECP cows versus 35% for 99 cows treated only with PGF2α. Results showed that a 3 mg dose of ECP 30-34 hrs after PGF2α treatment 1) can increase AI submission and pregnancy rates in unobserved estrus dairy cows judged by palpation to have a functional CL when AI'd based on detection of estrus, 2) can tighten the estrus response pattern of responding cows, and 3) but did not improve pregnancy rate results when cows were AI'd by appointment at 72-76 hrs after PGF2α.

I did not publish this research although I believe it demonstrates significant results for controlling estrus and ovulation with the combined use of PGF2α and ECP. Unfortunately, the work is now nearly irrelevant because of public health concerns regarding estradiol in the human food supply and the fact that ECP is no longer marketed for use in dairy cattle. Nonetheless, these findings were a rewarding culmination to 35 years of theriogenology research. I am pleased to have had the opportunity to share them with you today.

Graduate Students

Working with graduate students has probably been the most rewarding part of my career. I totally enjoyed the chance to help them search for goals, answers and their future. In fact most of these ladies and gentlemen gave more to me than I gave to them. Every one of them educated me in some way. I have been able to visit many places and do many things. With Ahmed Tibary, I saw great horsemanship and some of the world's most beautiful Arabian horses in Morocco. I saw beef production and the AI industry in Argentina with Miquel Fortin. I reviewed theriogenology curricula and graduate programs at 3 veterinary colleges in Thailand with Preeyphan Udompresert. By the recommendation of Juan Ramono, I visited dairy farms and gave CE lectures for veterinary practitioners in Uruguay. With translation help from Stephano Romagnoli, I lectured at the veterinary school in Pizza, Italy and to practitioners in Sardinia. From Ting Q. Zhang, I learned about China. When I started, my world was quite small but by these relationships it is now much more complete. I cannot list you all here but please know that I am grateful for the experiences we shared. Thank you!

Retirement and Beyond

Bovine theriogenology and academia have been very good to me and my family (wife Trish, daughter Ann, son Tony, daughter-in-law Sarah Beth, and granddaughter Avery). I cherish the memories. After 35 years it was time to walk away – while I could still hit a golf ball. Since golf season is short in Minnesota, I soon needed another activity. It has been surprising what a veterinary education and a theriogenology career prepared me for. For 6 years now I have done corneal excisions on organ donors as an eye procurement technician for the Minnesota Lions Eye Bank. This too has been an interesting and rewarding experience.

If you have not already done so, I encourage you to register as an organ donor, discuss your wishes with your next of kin and give the gift of life or sight to someone in need.

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2009 Theriogenologist of the Year

Ramblings of an itinerant theriogenologist

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Introduction

The invitation to prepare a written composition for this award creates a novel opportunity for a 'bully pulpit' from which to share my story. In that regard, I have chosen to briefly review my background, education, and activities, and as well to share the philosophical principles on which I operate.

It is an incredible honor and privilege to be designated as Theriogenologist of the Year! I am profoundly grateful to the selection committee, and to my colleagues who nominated and supported me. Furthermore, I am deeply indebted to all those who have helped me in ways big and small; it is no exaggeration to say that I accept this award on behalf of numerous persons who have been part of my world.

Early years and family

Life is a journey; what an incredible ride it has been for me! I had a very humble start on a small dairy farm near Edmonton, Alberta, Canada. I am the youngest of four children and am eternally grateful to my parents, John and Marge Kastelic, who taught me the value of hard work, honesty, and the pursuit of excellence. Although neither of my parents had the opportunity to complete high school, they have incredible wisdom and life experience. They strongly believed in the value of a good education, and they take enormous pride in the accomplishments of their children, grandchildren, and great-grandchildren. Furthermore, as they approach their 65th wedding anniversary, they are incredible role models of fidelity and long-term commitment.

My wife Rose and I have six children. Angela is a nurse, currently working on a Master's degree and Nurse Practitioner certification. Rachel has training as a child care worker, is married, and lives in New Zealand. Our next three sons are professional musicians; all are classically trained violinists. John 'migrated' to the viola, and is majoring in music theory and composition. Tony also switched to the viola and is majoring in viola performance and music theory. Gabe continues to play violin and will start his post-secondary studies in the fall. David is still in high school and is interested in the outdoors and farming. Rose chose to stay home to raise our children, and for sixteen years, has supervised the education of our children, including home schooling and cyberschooling (computer-based schooling). As the demands on Rose's time for educating our children have diminished, she is now pursuing a long-term goal of learning to play cello and will attend university part-time to pursue language studies.

As a teenager, I recall reading the advice that "If you prepare yourself appropriately, your way in the world will become very clear to you." Although I initially lacked the wisdom to understand the implications of that statement, I never forgot it. I certainly never imagined the opportunities and experiences that have come my way. I keep 'morphing' into the next phase of my life, from a student, to a veterinarian, scientist, editor, and writing consultant. Remarkably, my previous experiences prepare me for the next phase. I am unsure what lies ahead, except that I am incredibly excited, as I have every anticipation that 'the best is yet to come.'

Education

I became a veterinarian (University of Saskatchewan, 1982) and spent two years in private veterinary practice (primarily cattle). I was interested in specializing in dairy practice and I assured my wife that I would return to my alma mater to 'spend a couple of years and then get a real job again.' That plan was soon abandoned when I recognized that I needed training to the PhD level for long-term opportunities and job security. When I mentioned this to Prof. Reuben Mapletoft, he suggested that I go to the University of Wisconsin-Madison. My first reaction was to dismiss this as sheer folly, but the next day, with my wife's blessings and Reuben's help, I was on the phone with Prof. O.J. Ginther. I was soon accepted into his laboratory, and arrived in Madison in late June, 1985. In collaboration with Gregg Adams (we were classmates in both veterinary and graduate school), I worked on mares for one breeding season, but thereafter worked exclusively on cattle. The training I received at Madison has created life-long opportunities. In 1990, I completed my studies and started working as a Research Scientist for the Canadian federal department of agriculture (similar to USDA).

Professional activities

I passed the qualifying examination of the ACT in 1994; there were seven successful candidates that year, though I was the only one with a Y chromosome! I am enormously grateful to be a member of the ACT. Although my background and ongoing activities and contributions are different than most other diplomates, this is a very diverse group, and I have always felt very well accepted.

An invitation to present a lecture on ultrasonography in cattle at the Brazilian Embryo Transfer Society in 1995 was followed by a ten-month sabbatical (with my family) to Brazil in 1997, and subsequently several other visits to South America. I have also been able to make several trips to Europe, primarily Hungary. In 2007, I was deeply honoured to receive a Professor of Honorary Cause from the Faculty of Veterinary Science, Szent István University, Budapest, Hungary. Remarkably, this institution started in 1787, making it one of the oldest veterinary schools in the world.

From 2001 to 2008, I served on the ACT examination committee. This was an incredible experience, which has fostered cherished friendships. Furthermore, at breakfast during an exam planning meeting in Nashville, I casually mentioned to Mats Troedsson that 'being Editor of *Theriogenology* would be my dream job'; this ultimately resulted in a gracious invitation from the late Vic Shille to serve as his replacement. I travelled to Gainesville in early January 2003, and spent a few days with Vic as I assumed the position of Co-Editor-in-Chief. In this capacity, I get manuscripts from much of the planet (except Europe and Africa; those are handled by my colleague Co-Editor-in-Chief, Prof. Fulvio Gandolfi, in Milan, Italy). At present, my duties include receiving an average of six submitted manuscripts each week, deciding which ones will be sent to review, finding reviewers, interacting with reviewers and authors, and making decisions regarding the suitability of manuscripts for publication. I also do final edits on an average of at least three papers each week, including submitted manuscripts which I accepted, and proceedings papers which were guided through the review and revision by a guest editor.

From 2005-2008, the proceedings of the annual SFT conference were published in *Theriogenology*. For each of those four years, I had personal responsibility to do the final edits on the proceedings articles prior to publication. I really appreciated the efforts of the authors, reviewers, and guest editors for their involvement in this process. In addition, I was profoundly grateful for the many compliments that I received from members of the Society and College regarding the professionalism and high standard that was established with publication of these proceedings in the journal. I have every confidence that this will continue, now that the proceedings will be published under the auspices of the new journal being launched by the Society.

Science and scientific writing

My journal appointment provides experience, credibility, and funds to support my other passion; giving lectures and workshops on science and science writing, primarily to those for whom English is not their first language. We expect scientists to conduct and communicate science, but few graduate programs have formal training in scientific writing; I was profoundly lucky to be mentored by Prof. Ginther, an incredibly skilled and prolific writer. Furthermore, to get international recognition, it's almost mandatory to publish in English. Writing in your native language is difficult, whereas writing in a non-native language is profoundly challenging. Remarkably, despite the great need for training in scientific writing, especially for those for whom English is not their native language, there is a paucity of assistance available.

I have a substantial publication record (author or co-author of >100 peer-reviewed papers and >200 abstracts, proceedings, reports, etc.), but I have always felt that I am a better editor than a writer. I gave my first presentation on scientific writing at the veterinary school in Botucatu, SP, Brazil in 1997, followed by a presentation in Budapest in 2004. I have subsequently given presentations on scientific writing in these two countries, as well as seven others, in North and South America, Europe, and Asia. On several occasions, I have done these in collaboration with my wife Rose. Although English is her native language, Rose spoke primarily German before she attended school, has an undergraduate degree in French, spent one year attending university in France, and also has a Masters in Political Science. When we do writing workshops together, Rose covers the principles of writing in English, including sentence structure, punctuation, grammar, paraphrasing, and preparing outlines, whereas I cover the principles of conducting science and preparing manuscripts. We genuinely enjoy travelling and working together; most of the time, we are able to include some sightseeing into our trips.

Philosophy

My formal training is in veterinary medicine and reproductive biology, with only a few humanities options, and certainly no formal training in philosophy. In the paragraphs that follow, I have summarized some of my basic philosophies and guiding principles. It is noteworthy that these notes are derived in part from a brief presentation on ethics and philosophy that I usually include in a scientific writing workshop (when time permits). The primary purpose of sharing these ideas is not so that they are universally accepted and adopted, but rather to challenge others to contemplate these issues and formulate their own approaches.

Education, knowledge, and wisdom

Although education, knowledge and wisdom are clearly related, I view them as distinctly different. Veterinarians spend many years in school, learning a myriad of facts. Whereas knowledge is specific information, I really like the definition that education is what you remember after you have forgotten the details. Furthermore, I regard wisdom as the ability to apply knowledge. We all know many persons that are highly educated, but lack

wisdom. Conversely, there are many persons with a paucity of formal education that are extremely wise and have much to contribute.

The people factor

Although much of veterinary medicine is focussed on work with animals, most veterinarians spend considerable time interacting with people. Consequently, personal rapport and communication skills are very important, both for oral, as well as written communications. Although clinicians and scientists historically often worked as independent professionals, it is becoming much more common to collaborate with others. For a successful collaboration, communication is critically important, along with a clear understanding of responsibilities, expectations, and benefits. In general, long-term collaborations are usually successful ONLY if all persons involved contribute and derive a tangible benefit.

Personal vision

Although most companies and organizations have a mission statement, in many cases, it's debatable whether this mission statement has relevance in day-to-day activities. Regardless, I strongly encourage everyone to develop a personal mission statement, and to use it to guide your activities and decisions. My own mission statement is the essence of simplicity; my mission is to help people. Thus, I spend much of my day interacting directly with family, colleagues, coworkers, students, and members of society, and indirectly with people from around the world.

I recall the late Otto Radostits saying that if you want to get something done, you ask the busiest person that you know; they will usually agree to help, and will usually do so. Conversely, the person who has truly little to do will typically give you an extended litany regarding how busy they are, and they are highly unlikely to assist you.

It's critically important to establish goals; everyone should have short-, medium- and long-term goals. These goals should cover a broad range, from short-term practical ones, to others which are fanciful. Regardless of the nature of the goal, in the absence of a plan to achieve it, you have a fantasy, and not a dream. Furthermore, it is wise to be careful what you wish for, as it may come to pass!

Problem solving

Numerous times each day, all of us encounter problems, challenges, and requests for assistance. Although it's easy to feel completely overwhelmed, the key is to keep things in perspective. To cope with these demands, I employ a two-step approach. I first determine if this is something that I can address. Although there are many things that I dislike or disagree with, by recognizing that I have no ability to change them, I put them aside, and avoid considerable personal angst and frustration. For things that I can address, on the basis of urgency, importance, and my own schedule, I determine an appropriate time frame for my response. Although this approach may seem simplistic, it has enabled me to face a seemingly overwhelming barrage, put aside that which I cannot influence or control, and develop a manageable schedule of response for things which I can affect.

It's mostly attitude

It's been said that your life is approximately 10% what happens to you and 90% how you respond to it. In that regard, happiness is mostly a choice; you will be as happy or unhappy as you choose to be! Bad things happen to good people on a daily basis, and life is frequently not fair. There is a well defined series of reactions in response to tragedy; shock, disbelief, denial, anger, and eventually acceptance, the resolve to move ahead, and restoration of normalcy. There are two key issues regarding this series of reactions. Firstly, trying to avoid the steps, for example, trying to move ahead without grieving, are usually not successful. Secondly, although some events are so profound and tragic that you many never fully recover, prolonged angst and perpetually feeling that you are a victim can be very debilitating.

All of us know persons that are consistently upbeat and cheerful, often despite many challenges and difficulties. How do they do it? For myself, I make a conscious effort to adopt a positive outlook as my default mode. Furthermore, when confronted with adversity, I try to keep calm and maintain perspective; most things which initially seemed like mountains, are truly speed bumps when given sober second thought.

Dare to be different

It's been said that it takes 'different' people to make a difference. Furthermore, I recall one of my mentors telling me that "It's not conventional, but that never stopped us in the past!" Thus, I frequently take an unorthodox approach to many of my tasks and opportunities. Furthermore, I frequently encourage others to pursue novel approaches.

Professionalism

Veterinarians are highly trained medical personnel, with an expectation of being regarded by society as professional persons. As veterinary professionals, we are obligated to maintain a high level of knowledge and skill, and to provide services that are in the best interests of our patients and clients. However, in my view, professionalism is not merely conferred by accomplishment or education, but rather it is something that we earn in accordance with our conduct, including how we treat our patients, and arguably, more importantly, how we treat

others, including clients, staff, and colleagues. I firmly believe, that as professionals, we are held to a higher standard of conduct, in all aspects of our lives, both in our work and our personal lives. In that regard, unscrupulous behaviour in our personal conduct results in a substantial loss of credibility in our work.

Work-life balance

As the world becomes more interconnected and complicated, the overall pace of our lives seems to increase. Many working people have challenges maintaining a healthy work-life balance; veterinarians are no exception, and for many, emergency calls may make it even more difficult, particularly for those who have few or no associates who share emergency duty. Although there are many articles and pundits who claim that 'you can do it all,' in my view, that is not realistic. I firmly believe that everything has consequences, both good and bad. For example, a speaking engagement in a foreign country provides me an opportunity to help others, to meet new persons, and to see a new place. However, it also means time away from my responsibilities and activities, both personal and professional. Although communications make it possible to stay in contact, for many aspects, they are not a satisfactory substitute. Thus, it is critical to honestly consider the true 'net benefit' of any action or decision. Furthermore, it is foolhardy to endlessly postpone commitments or actions, as they may never happen. We all have to take personal responsibility for our own health and wellbeing, both mental and physical, including development of appropriate leisure activities. The consequences of failing to address these needs can be truly tragic.

21st century

The world has changed dramatically in the last few decades, and if anything, the pace of change is accelerating. In its current iteration, the world is an exciting, but complicated place! Communication and travel are 'shrinking' the world; we have unprecedented opportunities to share ideas, knowledge, and experience with others, both electronically, or within a few hours of travel, we can be virtually anywhere on the planet. Thus, there are literally endless possibilities for all of us, in particular for youth and young adults. Concurrently, the challenge is to carefully sift through all the 'noise' and 'fluff', and to capture the real 'nuggets', and ultimately make good choices and decisions, based on correct and reliable information.

In step with the rise of the 'information age', there has been an increased emphasis on marketing. I recall reading the liner notes on a music CD, where the artist acknowledged her 'marketing team.' Thus, her incredible commercial success was due not only to her substantial talent, but concurrently to a clever group of people with detailed knowledge of marketing. In my view, many other entities (not just musical groups, but the world in general) have very little apparent talent, substance or inherent value, yet they are incredible commercial successes, due primarily to the role of marketing. I think there is an important lesson here for all of us. We live in a highly competitive world and we need to learn to market ourselves, including our knowledge, skills, and our opportunity to provide a service, product, or somehow to add value. I recommend a two-step approach. First, identify your inherent skills, ability, and knowledge, and then work very hard to increase and improve those; to truly achieve to your potential. Secondly, market yourself, your skills, and abilities. We have all done this; for example, we had to get good grades and experience to gain entry to veterinary school. As information becomes more readily available and the overall levels of competition continue to rise, we all need to pro-actively enhance our resumes, our knowledge, skill sets, and abilities, so that we can remain competitive. Furthermore, in my view, it's not necessary to put others down to gain a competitive advantage; we need to 'put ourselves up', through ongoing evaluation and renewal.

Conclusion

I reiterate my profound gratitude to the selection committee for choosing me to receive this award, to all of those who have helped me along the way, and especially to my family, who provide ongoing love, support, and indeed, a reason to live. It seems appropriate to end with a quote from Winston Churchill: "We make a living by what we get, but we make a life by what we give."

Legal and ethical veterinary compounding

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Food and Drug Administration

The Food and Drug Administration (FDA) first made a distinction between human and veterinary-labeled drugs in 1968. As with most regulations since that time, the distinction reflected a concern for human food safety. At that point in time, an animal drug was adulterated if used in an extra- label manner. Consequently, veterinarians were not permitted to use any drug except according to the label claim, without being in violation of both criminal and civil law. In an ideal world, pharmaceutical dosage formulations would exist to treat all disease conditions in humans as well as all species of animals. The economic reality does not permit the establishment of numerous pharmaceutical solutions for most disease states and relatively few pharmaceuticals have been developed for animal species. The development of a new animal drug typically averages ten years and cost $40 million. This combined with the narrow profit margins for most products makes for a challenging market place.

By definition, a New Animal Drug is "any drug intended for use in animals other than man...".[1,2] Veterinarians must by necessity on occasion utilize products that are compounded to meet a specific medical need. However, the AVMA guidelines for pharmaceutical compounding state that compounded products "may be used only when a need has been established and FDA-approved products are not available or clinically effective."[2,3] While FDA-approved products are extensively tested for efficacy, quality, purity, strength, bioavailability, and stability, the testing that compounded formulations are subjected to is extremely variable.

The FDA's Center for Veterinary Medicine (CVM) allows for the compounding of animal drugs under the 1994 Animal Medicinal Drug Use Clarification Act (AMDUCA).[2] This Act became a law in 1996 and extended the veterinarian's authority to use human drugs in an off-label manner, including the right to compound with the use of FDA-approved dosage forms. AMDUCA does not allow for the compounding of drugs from bulk active substances, and any products resulting from such substances are considered new animal drugs and are subject to the FDA drug-approval process.

The FDA Modernization Act of 1997 allows for the compounding of human drugs from bulk chemicals as long as the bulk substance is an ingredient of a currently approved product that appears on an FDA list of drugs that can be compounded.[3] It is important to note, however, that ingredients that are on the list of bulk substances withdrawn from the market for safety reasons may not be compounded. Human compounding regulations are sharp contrast to current animal compounding regulations. These regulations become an important consideration for veterinarians who out-source compounding to pharmacies that may compound exclusively from bulk active chemicals. Bulk chemicals are defined as active ingredients used in the manufacture of finished dosage forms of the drug. Bulk chemicals are also referred to as the active pharmaceutical ingredients (API). Compounding from bulk is allowed in instances where the health of the animal is at risk and there are no other remedies.

Drug: The FDA defines a drug as any substance, food or nonfood; intended for diagnosis, cure, mitigation, or prevention of disease in humans or other animals; intended to affect body structure or function; or any substance administered by injection.[3] This broad definition effectively means that any substance used to treat an animal can be considered a drug.

Veterinary Compounding

Drug compounding can be defined as the art and science of mixing ingredients, which may be active, inactive or both, to create a specific dosage form to meet a particular patient's needs. For example, mixing two injectable drugs is compounding. Compounding can be performed by a veterinarian, or by a pharmacist upon receipt of a veterinarian's prescription for a particular patient. A veterinarian must have a valid veterinarian-client-patient relationship in order to legally prescribe or prepare a compounded product. Federal regulations require that legally compound drugs meet the following criteria:[3]

- A valid Veterinarian-Client-Patient relationship (VCPR) must exist.
- The health of an animal must be threatened or suffering or death may result from failure to treat.
- There must be no FDA-approved, commercially available animal or human drug that, when used as labeled or in an extra-label fashion in its available dosage form and concentration, will appropriately treat the patient.
- The product must be made from an FDA-approved commercially available animal or human drug.

- The product must be compounded by a licensed veterinarian or a licensed pharmacist on the order of a veterinarian within the practice of veterinary medicine.
- The compounded product must be safe and effective.
- The amount of product compounded must be commensurate with the need of the animal identified in the VCPR-based prescription.
- For animals produced for human consumption, the veterinarian must establish an extended withdrawal interval for the compounded product and ensure food safety. Compounding is not permitted if it results in violative food residue, or any residue that may present a risk to public health.
- No drug may be compounded for food animals from drugs listed on the prohibited list.
- Veterinarians must comply with all aspects of the federal extra-label drug use regulations including record-keeping and labeling requirements.

Pharmacies specializing in veterinary compounding have been growing exponentially aided by the ability to reach a larger number of consumers via the Internet. Many commercial websites market directly to the owner offering subjective treatments based on testimonials and compounded therapies that are not permitted by the criteria established for compounded drugs. Currently, the FDA does not have the resources to enforce these regulations; however, veterinarians should be aware that abuse of these regulations can result in legal action (e.g., FDA warning letters, confiscation of inventory and other enforcement action). The FDA has determined that it will seriously consider taking action when the scope and nature of activities of veterinarians and pharmacists raise concerns normally associated with a drug manufacturer resulting in significant violations of the new animal drug, including adulteration, or misbranding.

Compounded drugs are not the same as generic drugs. Generic drugs are FDA-approved. To receive FDA approval, generic drugs must demonstrate bioequivalence to the "pioneer brand name" drug. Generic drugs can be identified by the ANADA number on their label and by cross-checking with a drug reference found in the FDA Green Book of Approved Animal Drug Products. In contrast, compounded drugs are extemporaneously prepared products that lack FDA approval.

The idea is that compounded drugs with their possible inadequacies are better than no drug at all and suitable for a small patient population. Equine practitioners using compounded products are put in a position of evaluating the integrity of the compounding pharmacy as well as the quality and consistency of the pharmaceuticals they produce. Lack of regulatory approval means that not all veterinary compounding pharmacies follow Good Manufacturing Practices (GMPs) Guidelines simply because they are not required to. In some instances, loose oversight has allowed negligent compounders to prepare products from unregulated raw materials with no quality standards. Other compounding pharmacies distribute medication without a valid prescription. Veterinarians are schooled on quality patient care but few pharmacists are.

Veterinarians who frequently use compounded products would be well advised to learn more about pharmacy issues related to veterinary medical therapy.

For example:

- It is illegal to compound a specific product when there is an approved drug form of that specific product, except to make a different dosing form. However, the approved product must be used to make the compounded new dose form.
- It is illegal to mark up prices on compounded drugs
- As a veterinarian, if you use a compounded product, you assume liability for any adverse effects or efficacy failure.
- Drug manufacturers are required to carry product liability insurance, pharmacies are not
- It is illegal to place expiration dates on compounded products
- It is illegal to have a drug compounded in order to obtain the drug at a lower price

Problems and concerns regarding the use of compounded drugs

Omeprazole . This study was undertaken to determine the efficacy of commercially available omeprazole paste and a compounded omeprazole suspension to heal gastric ulcers.[4] Results from this study suggested that while administration of the commercially available omeprazole formulation was effective in promoting healing of gastric ulcers in horses, administration of the compounded omeprazole suspension was ineffective. Differences in the

source of omeprazole and partial inactivation of omeprazole by the vehicle or by gastric contents following administration of the suspension were cited as possible reasons for the poor results seen with the compounded product.

Pharmaceutical equivalence. The pharmaceutical potency of compounded preparations of ketoprofen, amikacin and boldenone were compared with commercially available FDA approved products.[5] The FDA requires any manufactured pharmaceutical to have a concentration (potency) of not less than ±10% of the expected concentration as stated on the product label. The results of this study found that 11 of the 22 compounded products failed to meet the FDA requirement for potency. Of the eleven products that failed to meet the FDA standard, the range in percentage potency was as low as 50% and as high as 150% of what was stated on the prescription label.

Compounded clenbuterol. In 2006, concentrated counterfeit clenbuterol was determined as the cause of death for several Thoroughbred horses in Louisiana. The compounded clenbuterol solution was analyzed and found to be extremely potent, ~70 times greater than the FDA approved commercial product Ventipulmin® (Boehringer Ingelheim Vetmedica, Inc, St. Joseph, MO USA). The source of the compounded clenbuterol solution was not determined but the trainer stated the product was "...just like Ventipulim® but cheaper...".

Compounded chloramphenicol palmitate. Six owners sued a New Jersey veterinary pharmacy alleging that a defective antibiotic led to the deaths of three horses, including Saratoga County and Egg Head, both stakes winners. They alleged negligence, breach of warranty, and strict products liability against the pharmacy involving the antibiotic product chloramphenicol palmitate, that they claimed necessitated the euthanasia of three horses. The case cites improper design, manufacture, compounding, formulation, mixing and/or labeling, (which) "led to the slow, painful demise of these horses." Following a two year legal battle the case was dismissed by the United States District Court in New York.

Conclusion

Products are required to treat hundreds of conditions and diseases in dozens of species. Compounding of drugs for use in animals is a necessary and beneficial component of veterinary practice. Licensed veterinarians may legally use or dispense prescription drug products ONLY within the course of their professional practice where a valid VCPR exists. FDA Compliance Policy Guides permit licensed practitioners to manufacture, prepare, propagate, compound or process drugs during the regular course of business, as long as the compounded product is NOT a new animal drug.

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Orchitis and epididymitis

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Abstract

Inflammation of the male testis and/or epididymis produces canine and feline subfertility and infertility. Causative agents include bacteria, trauma, non-infectious immune-mediated response, prostatitis, urinary tract infection, or an accompanying endocrine disorder. Each possible etiology should be evaluated to both determine the reason for the abnormal reproductive function and to justify its treatment.

Keywords: Orchitis, epididymitis, dog, cat, *Brucella*

Introduction

In the male, loss of reproductive performance is sometimes first observed as failure of conception in the female to whom the male had been bred, or as a painful attempt during a natural breeding. This change in behavior may be the most apparent sign in an otherwise asymptomatic male. A male who historically exhibited normal breeding behavior may become unwilling to mount and gain intromission. An affected male may start scrotal licking to the extent of hair loss and scrotal dermatitis. Multiple etiological agents are possible for such behavior. Only after obtaining a good history, performing a thorough physical examination, and choosing the appropriate diagnostic tests can the underlying reason for a behavioral change, pregnancy failure in bred females, or purulent preputial discharge be determined. Subsequent therapy can then begin which may or may not maintain the male's potential to be a satisfactory breeder. Orchitis and epididymitis can occur separately or in concert since each organ has close anatomical positioning and connection to a common excurrent duct system. Inflammation of one structure can lead to inflammatory response of the other and thus the term orchiepididymitis or epididymo-orchitis is used in veterinary literature.[1-4] This condition is rarely found or perhaps diagnosed among tom cats.

Anatomy

Body weight and breed dimensions should correlate with testicular weight, volume, width and, therefore, quantity of ejaculate. Each testicle is positioned obliquely within the scrotum of the stud dog and tom cat. The head of the epididymis is attached to the cranial aspect of the testis, the body lying on the dorsolateral surface, and the tail fixed to the caudal end by the ligament of the tail of the epididymis or former gubernaculum testis.[5] The epididymis continues as the ductus deferens within the spermatic cord, which also encloses the testicular artery and vein, pampiniform plexus, cremaster muscle, and lymphatics. The cord is wrapped on the outside by the visceral vaginal tunic and overlaid by the parietal vaginal tunic as it passes through the inguinal canal. Deep to these two tunics, the testis is also covered by a thick, white, fibrous, tightly adherent capsule called the tunica albuginea.

The body of the testis or testicular parenchyma is subdivided into compartments of seminiferous tubules by connective tissue septae. Spermatogenesis occurs within these seminiferous tubules. The sections of tubules feed sperm into a collection of spaces and ducts called the rete testis. A band of connective tissue called the mediastinum testis splits the testicle longitudinally and serves as the entry and exit point for testicular blood vessels and lymphatics. From the rete testis, sperm cells then move into and through the head, body and tail of the epididymis where spermatozoal maturation and storage occur.

Descent of the testicles into the scrotum occurs in a similar pattern for the dog and tom cat. As the gubernaculum shortens, each testis passes through its respective inguinal canal and ring. When this passage occurs, the testes is already covered by the visceral tunic and then overlaid with the outer or superficial parietal tunic. Completion of this process usually occurs prior to birth in the normal male. However, male cats have been noted to have testicular movement back and forth through the inguinal canal after birth and prior to puberty.[6]

Each testis is partitioned into three sections. The interstitial portion contains the Leydig or interstitial cells, blood vessels and some support tissue. This section functions to provide hormones and nutrition to its respective area. The second or basal compartment is comprised of Sertoli cells and undifferentiated germ cells called spermatogonia. The third and innermost adluminal compartment houses the developing stages of spermatozoa and is separated from the basal section by the immunologically sensitive blood-testis barrier.

Etiology

Infectious

Bacteria enter the testis or epididymis through several routes: from an ascending source through the penile urethra, from descending flora within the adjacent bladder or prostate, and hematogenously. The primary bacterium associated with canine infertility is *Brucella canis,*[7] however other *Brucella* spp. (eg. *Brucella abortus)* have been cultured from the epididymis in the canine.[8] Additional bacteria are hemolytic *E. coli*, *Proteus vulgaris*, *Staphylococcus* spp. as a natural skin contaminant, and *Mycoplasma* spp.[3-9] Also the canine distemper virus[10] with its cytoplasmic and intranuclear inclusions, granulomatous epididymitis from mycotic *Rhodotorula glutinis*,[11]

Blastomyces,[12] human tuberculosis,[3] Rocky Mountain spotted fever,[4] and canine ehrlichiosis (Lyme disease) have been reported as potential causes. The distemper virus is spread venereally to susceptible bitches which acquire an endometritis.[3] *Mycoplasma canis* is a natural component of urogenital and respiratory mucosal surfaces in the dog and cat[13] and can cause purulent prostatitis and epididymitis. A bacterial urinary tract infection can incite orchiepididymitis since there is potential for retrograde or reverse urethral pressure through the ductus deferens. Bacterial infections can occur following injuries from pelvic fracture and surgical resection of ileum.[3] Unusual reports occur when certain stud dogs still sire litters despite heavy bacterial growth of *Klebsiella pneumoniae* and *Streptococcus* after antibiotic treatment for a preliminary diagnosis of epididymitis.[14]

A stray cat that was positive for feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) had chronic necrotic and fibrinous orchitis.[15] Tuberculosis was reported as the cause for a reproductive infection of tom cats in foreign countries.[16] *Brucella* spp.[7,17,18] and periorchitis from feline infectious peritonitis (FIP) were noted as well. Cats are resistant to infection with *Brucella canis*.[19]

Testicular injury from a bite wound or puncture results in injection of bacteria or viral particles into the animal, followed by entry into the venous blood and initiation of an inflammatory response. The cellular influx causes swelling and edema of the immediate area. White blood cells are attracted to the site and begin phagocytosis. The increased blood supply soon spreads a local infection systemically. If the animal becomes septicemic with bacteria such as hemolytic *E. coli*, effects of endotoxemia on the kidney impair glomerular filtration rates. As endotoxemia ensues, the animal becomes febrile. A local thermal effect on the ipsilateral testis diminishes spermatogenesis and promotes agglutination of sperm which turns into oligospermia, anesthospermia, or azoospermia. As the condition becomes chronic, testicular atrophy results. Bacterial ascension from proximal prostatic secretions or a urinary tract infection can initiate orchitis and epididymitis.

Non-infectious

Non-infectious, atraumatic orchitis or epididimytis occurs with the entry of sterile urine through the ductus deferens into the testicle. Blunt force injury to the abdomen, such as happens from an animal being hit by a car, can cause urine to abnormally flow from a full bladder to the testicles.[3]

In humans, even temporary duct blockage secondary to prostatitis can lead to immune-mediated infertility.[20] The blood-testis barrier was described in detail by Amann in 1989.[21] The blood-testis barrier is comprised of tight junctions between adjacent Sertoli cells resting on the basement membrane. Any breakdown of this barrier, from trauma, infection or an inflammatory response, breaches the immunologically protected status of the testis. This affront allows the immune system to react to the antigenic insult. Sometimes the reaction produces a sperm granuloma at the puncture point. The attraction of plasma cells and lymphocytes into the testicle does produce a residue of immunoglobulins within the seminiferous tubules. The loss of testicular function by the tubules, Leydig cells and impaired vascular flow results in reduced sperm development. Clinically, immune-mediated infertility will be manifested by oligospermia or azoospermia. Immune-mediated orchitis can be temporary or permanent.[20]

Similar to that observed in humans, immune-mediated disease has been associated with infertility in the dog. The incidence of lymphocytic orchitis in dogs is correlated with lymphocytic thyroiditis, a heritable trait in Beagles.[22,23] Males with this autoimmune disorder had lesions of tubular degeneration, atrophy and lymphocytic orchitis. Therefore, a history of reproductive failure in a stud dog that has been diagnosed with other endocrine diseases should prompt further analysis.

Pathophysiology

The blood supply to the testes and epididymides comes from the testicular artery and the artery of the ductus deferens, respectively. Both structures are influenced by hormones, both play an important role in spermatogenesis, and both tissues are altered by inflammation and antibody formation. Existing infections within the bladder or prostate gain entry into the ductus deferens by retrograde route. Inflammation from regional lymph nodes reaches the testis or epididymis by lymphatic drainage and then into the blood stream. Epididymal occlusion may appear secondary to an infection. The more proximal the blockage to the adjacent testis, the worse the damage to that testicle.[24]

Viral agents such as feline coronavirus are engulfed by and replicate in macrophages. These macrophages then travel to target organs and mix with lymphocytes to form a fibrin layer on the tunica albuginea (i.e., periorchitis), a finding consistent with FIP.[15] Since the parietal and vaginal tunics are continuations of the peritoneal cavity, seeding from a contaminated surgical site or traumatic injury can extend infections into these reproductive organs. Small abscesses within the lumen or testicular parenchyma increase in size and extend fistulous tracts through the scrotal wall. Adhesions develop and block tubular patency. Gangrenous inflammation occurs within the unyielding tunics, which inhibit testicular mobility and increase scrotal temperature. Inflammation of the spermatic cord results in vascular compromise, tissue necrosis and eventually testicular atrophy.[3]

The interstitial compartment houses immune cells that maintain the testis in an immunologically secure location. Spermatogonia within this compartment are protected from an autoimmune attack. In addition to the blood-testis barrier, a multitude of factors establish this privileged immune status. Androgens influence the inhibition of proinflammatory cytokines.[25] Pro- and anti-inflammatory cytokines regulate testicular function relative to both spermatogenesis and steroidogenesis.[26] Pro-inflammatory cytokines such as interleukin and anti-inflammatory cytokines are linked to testicular development.[27] Therefore, an insult from infection or trauma induces production of these respective regulatory proteins and likewise disrupts testicular function. Timed progression of focal or diffuse inflammation in testicular tissue eventually results in loss of seminiferous tubules by replacement with connective tissue fibrosis. The clinical outcome of this sequence is manifested as infertility.

Another source of immune protection in the testis is the resident population of macrophages. Macrophages assert cytotoxic and phagocytic activity against infection through the cellular inflammatory response.[28] For example, *Brucella canis* embeds itself within macrophages that ultimately seek steroid-dependent organs such as the testis and epididymis.[7] These immune and inflammatory components combat and modify normal internal testicular functions that correspond to owners' complaints of nonpregnant matings, scrotal swelling or pain.

Clinical signs

Historical information provided from the breeder client often supplies the first clue to the diagnosis of orchiepididimytis. Breeder clients are conscientious and notice conception failures. Owners or handlers observe stud dogs not wanting to ejaculate, or an enlarged scrotum while grooming for a show or field trial. The client may report previous urinary or reproductive tract infections, an increased licking of the scrotum, an obscure hindleg lameness, stiff or altered gait, purulent preputial discharge, or palpable unilateral testicular atrophy. All prior medical problems, treatments, hormonal adjuvants and products should be chronologically recorded for review.[29] As one source commented, a client's conclusion may be 'infertility in an otherwise asymptomatic dog'.[2] Additional signs include lethargy, anorexia, fever of unknown origin, inappetance, and vomition. Physical findings indicate a swollen scrotum and testicle or epididymis, primarily unilateral enlargement, but it can be bilateral. The testis is asymmetrical; the scrotum is reddened or hyperemic and hyperthermic. The dog licks the area causing a 'lick' granuloma or scrotal dermatitis and exhibits a level of discomfort and pain during palpation of the testicles. If the dog resists or is reluctant for digital examination, tranquilization might be necessary. The expression of pain can be acute, episodic and increase with intensity during a natural cover or manual collection. A decrease or loss of libido is evident. One older male had a progressive bilateral alopecia and feminization syndrome associated with acute and chronic epididymitis.[30]

Digital palpation may distinguish swelling of the head, body and/or tail of the epididymis and testes. The swollen area often has a soft, doughy texture in acute cases, and a firm, fibrotic consistency in chronic cases. A small puncture wound may be discovered with or without draining purulent exudate or concurrent orchitis. A spermatocoele, sperm granuloma, or hematoma within the tunic linings or in the vaginal cavity may be found. A serosanguineous or purulent discharge from the dog's preputial orifice may be present.

In summary, the typical clinical signs exhibited with prostatitis include a blood-tinged ejaculate, hematuria, and difficulty in defecation. Massage of the prostate per rectum may aid in detection of a purulent discharge from the urethra.[3] However, like prostatitis, orchioepididymitis can present with similar signs: altered hindlimb gait, lethargy or pain. To help differentiate prostatitis from orchioepididymitis, digital examination would reveal symmetrical or asymmetrical distension of the inflamed prostate.

Diagnosis

A thorough anamnesis should be recorded. Historical information should include details of prior reproductive attempts, onset and duration of signs and subtle changes in behavior or clinical signs. A chronologically ordered medical history is beneficial. Notations are made of diet, supplements, deworming program, schedule of vaccinations and past medications or surgical therapy. Potentially confounding effects of age, temperament and conformation on behavior or locomotion need to be noted. A physical examination, beginning with a TPR, should conclude with a digital evaluation per rectum of the prostate for size, symmetry, consistency, and pain response. Palpation of each testis for size, shape and consistency can often discern a soft, acute onset condition from a firm, nodular chronic problem. Likewise, palpation of the epididimydes can aid in the identification of unilateral or bilateral testicular atrophy based upon the relative prominence of epididymal structures. If severe, acute pain is shown during examination, the primary differential diagnosis is torsion of the spermatic cord. If the scrotum itself is swollen, the examiner then must determine whether it is an intratesticular enlargement or an extratesticular disorder. Ultrasonography would assist in differentiation of the location and tissues involved.[31] A swollen scrotum should be distinguished from swollen testes.[32] Symmetrical scrotal enlargement occurs in orchitis, hydrocele, and torsion of the spermatic cord. Asymmetrical scrotal shape results from neoplasia, varicocele,

epididymitis, abscess, or hematoma. An ultrasound-guided cystocentesis can be performed and urine submitted for cytology and culture.

In all cases of orchioepididymitis, a screening test for *Brucella canis* should be submitted with other samples for clinical pathology. A caution should be issued at that time to client and clinic regarding minimization of potential for human exposure to this zoonotic disease until the results are confirmed.

For the breeding soundness evaluation, testicular measurements with a caliper document any change in size or shape. The semen collection should be fractionated into separate samples for determination of sperm motility, morphology, concentration, cytology, culture and sensitivity, and pH. Sperm can also be placed in extender for computerized analysis. In azoospermic samples, an alkaline phosphatase value from seminal plasma of the second fraction would confirm epididymal patency or blockage. In cases of oligospermia or azoospermia, retrograde ejaculation of sperm into the urinary bladder can be diagnosed by the presence of spermatozoa in urine obtained post-collection.[9,23] A stained cytology slide of the third fraction containing ample bacteria and greater than three to five neutrophils per high power field (100X) is diagnostic for prostatitis. Further imaging or follow-up evaluation is indicated. The semen evaluation yields a decreased number of sperm and an increased amount of abnormal morphology from increased agglutination. Bacterial epididymitis causes sperm acrosomal degeneration with subsequent loss of the plasma membrane. Affected sperm cells cannot penetrate the zona pellucida of the oocyte. Infertility results.[34]

Except during the active process of ejaculation and urination, seminal fluid normally moves from the ductus deferens into the bladder.[21] The prostatic fluid travels cranially into the bladder by normal urethra pressure.[35] Therefore, bacteria from the ductus can produce an ascending prostatic or urinary tract infection (UTI). Culture of a urine sample obtained by cystocentesis would identify the causative bacteria, including the opportunist, *Mycoplasma canis,* if the specimen is cultured on appropriate media.[13]

Careful manipulation of the scrotum and its contents is useful in the identification of a puncture wound or laceration, a draining tract, a change in skin thickness, altered sensitivity, differentiation of intratesticular or extratesticular origin, or the soft core of an abscess as one sequela of orchioepididymitis. Fibrosis and scrotal adhesions restrict testicular mobility, and with degeneration, the consistency changes from soft, edematous tissue to a firm, hard, smaller and fibrotic testis.

Ultrasonography (US) provides a rapid, non-invasive imaging tool beneficial for diagnosis, prognosis and a therapeutic plan.[31,36] With US, abnormal testicular architecture can be differentiated from the diffuse, hypoechoic pattern imaged in normal testicular parenchyma.[37] Fluid in testicular cysts, a spermatocele, or an abscess can also be detected.[37] In humans, the most common diagnosis for a swollen, painful scrotum is epididimytis, and it is most commonly diagnosed on ultrasound examination when viewed as increased vascularity in a testis of otherwise normal architecture.[38] When a dog presents with an acute onset of scrotal pain and swelling, color flow or power Doppler US imaging can distinguish between the lack of arterial and venous perfusion caused by torsion of the spermatic cord and the hyperemic blood flow from an infectious agent.[39,40] Following an incident of testicular torsion, Doppler can visualize disrupted blood flow within the cord and altered echogenicity from testicular infarction. Doppler US is helpful in the localization of a lesion in either the tail of the epididymis, the spermatic cord or tunics, or the testis proper. Neoplasia, either Leydig or Sertoli cell tumors, may have hypoechoic and hyperechoic areas that are usually well-defined.[4] Testicular tumors have an irregular, lobulated contour versus orchitis with a moderately enlarged, oval shaped, smooth textured testicle.[4,39] Tumors of the epididymis are rare.[41] However, documentation in human cases is more widely reported.

One author suggested using fine-needle aspiration (FNA) from the caudae epididymides to collect diagnostic samples under sterile conditions from infertile males for cytology, histopathology, culture (aerobic, anaerobic, and *Mycoplasma*).[42] Fine-needle aspiration has been used to confirm obstructive lesion or maturation arrest for oligospermia and azoospermia.[43] Ultrasonography can select which patient would benefit from this procedure and avoid inherent risk or immunological consequences.[36] The accuracy and placement of the needle can be improved through ultrasound guidance as well. While a FNA sample for a cytology smear is presumptive for diseases such as lymphocytic orchitis, a testicular slice biopsy will confirm the diagnosis. It may be advisable to weigh the short-term benefit versus the long-term risk since adverse effects may develop later. Focal hemorrhage, interstitial fibrosis and tubular atrophy can occur at a biopsy site, but adjacent tissue and semen quality may remain within normal limits.[44,45] With either method, there is risk for sperm granuloma formation or antisperm antibody production as a result of 'foreign' protein leaking from the point of surgical extraction through tunics, cavity, parenchyma and blood-testis barrier. One would obviously not perform a testicular biopsy or risk anesthesia in the presence of inflammation.[2]

Prostatic disease can be verified by anamnesis, digital examination, culture and sensitivity, cytology of the third fraction or prostatic wash, ultrasonography, and ultrasound-guided aspirate or biopsy.

If an orchidectomy of a diseased testis is performed, samples obtained at surgery should be cultured for aerobic bacteria, *Mycoplasma spp.* and fungi, and tissues should be sent for histopathology.

Pathology

Epididymitis is one of the more common inflammatory diseases of the reproductive tract. The usual route of entry for pathogens into the epididymis is via an ascending or retrograde mechanism. Trauma to the scrotum combined with endotoxin-producing bacteria such *E. coli* results in septicemia. The epididymis lacks a natural local immune system. Lymphocytes or plasma cells are recruited after the initial insult from infection occurs. Edema progresses to an abscess or sperm granuloma formation. Fibrous bands appear between the tunics, which are extensions of the peritoneal cavity,[3] and the epididymis. In time, these adhesions lead to testicular atrophy and firm, nodular ducts. Scrotal swelling is caused by the accumulation of fibrinopurulent exudate in the tunic cavity. This cellular response includes lymphocytes, neutrophils and macrophages. Extensive luminal fibrosis and spermatocele can occlude the ducts even after successful treatment and decreased signs of inflammation. Disruption of the blood-testis barrier initiates an immune-mediated reaction and results in lymphocytic infiltration. Inflammation of the seminiferous tubules causes degeneration. The inflammatory response within the testicular parenchyma is suppurative, with abscessation and possible fistula formation to the exterior scrotum.[17] Lesions in a cat with orchitis were consistent with vasculitis, increased fibrinogen and infarction from simultaneous viral infections.[15] Multifocal necrotic granulomatous inflammation of the pleura, lung, eye and lip were found.

Treatment

Similar treatment protocols are suggested for both orchitis and/or epididymitis. Once the cause is known, an appropriate regimen can be instituted. Antimicrobial therapy is started based upon results of the culture and sensitivity. A minimum duration of treatment is three to four weeks. Concurrent sexual rest is implied. Another culture should be obtained one to two weeks after the antibiotic has been discontinued. Resolution of epididymitis requires the correct antimicrobial choice, dosage and length of administration. Too often, failure of client compliance alters the outcome when treatment is stopped prematurely. Within days, owners may supplement with their choice of medication in addition to or in place of the one prescribed. Immunosuppressive drugs have been used on a short-term basis to treat immune-mediated disease. However, continued use of immuosuppressive agents may adversely affect spermatogenesis and lead to infertility.

Owners should be apprised of possible outcomes and expense. Serial monitoring can document changes in semen parameters and allow for alteration of therapy if necessary. Brucellosis management requires immediate quarantine and testing of dogs having had contact with the confirmed case. Positive dogs are removed from the premises and are either euthanized or retested until two negative results have been obtained. Antibiotics will decrease bacteremic signs and neutering does eliminate the target organs, but the chance for relapse is probable. Since brucellosis is zoonotic, exposure to pregnant women, children and immunosuppressed people, in particular, should be avoided. Treatment for unilateral epididymitis offers a better recovery rate; bilateral infection has a poorer prognosis for a return to satisfactory reproductive performance.[23]

The treatment of choice may be surgical excision of the infected tissue. Relief is immediate and the potential for fertility may be salvaged. Hemicastration or orchidectomy of the diseased testis not only removes the source of inflammation or benign neoplasia but also decreases the potential for thermal insult on the unaffected, contralateral testis. Following unilateral orchidectomy, an immediate reduction in sperm output was followed by increased spermatogenesis and compensatory hypertrophy by the remaining testis.[46] If the preliminary histopathology confirms malignancy or an irreversible condition, complete castration may be recommended for the animal's welfare. If the cause can be reversed, then the male may recoup fertility. If the primary problem is torsion of the spermatic cord, an infectious disease or immune-mediated disorder which resulted in secondary damage, any resolution of pre-existing orchioepididymitis may be irrelevant. The progressive fibrosis within the tubular compartments would gradually prevent spermatogenesis and steroidogenesis. An epididymotomy has been suggested as a treatment for bacterial epididymitis in men.[47]

Prevention

To control exposure and transmission of disease, screen new arrivals to a kennel, test animals at least annually for *Brucella canis*, disinfect facilities regularly especially the whelping environment, reduce fomite contamination during breeding, and employ artificial insemination when possible. Judicious use of antibiotics in the female during her estrous cycle and breeding is important to avoid bacterial resistance and birth defects. Good management includes periodic monitoring for urinary tract infections and early detection of subfertility or infertility, followed by an accurate diagnosis and prompt treatment.

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Antibiotic use in prostatic disease in dogs

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Abstract

Knowledge of pharmacokinetics of currently available antibiotics permits veterinarians to choose antibiotic therapy based on microbial culture and sensitivity results and ability of various classes of antibiotics to readily distribute through prostatic tissue. This may replace other diagnostic tests used to support antibiotic choice, such as measurement of pH of ejaculated prostatic fluid.

Keywords: Prostate, prostatitis, fluroquinolone

Introduction

The prostate is the only accessory sex gland of dogs. It is a retroperitoneal organ, encircling the neck of the urinary bladder. It is bilobed with a palpable median raphe. In smaller breed and younger dogs, it is palpable per rectum. It may be difficult to palpate per rectum in large or giant breed dogs of any age, and in dogs with age-related increase in prostate size and subsequent cranial positioning of the prostate and urinary bladder.

The prostatic capsule is thick and contains smooth muscle fibers, some as an extension of muscle fibers from the wall of the urinary bladder. Some believe there is no such thing as a true prostatic capsule; one study evaluating prostate histology after prostatectomy in men with prostatic carcinoma instead defined a fibromuscular band of varying thickness that was an inseparable continuation of the prostate stroma.[1] The prostatic capsule may be more of a functional than a histologic entity.

The canine prostate is made up of lobules of secretory tissue separated radially by bands of connective tissue. These lobules are larger and contain more secretory tissue in the dorsal and lateral areas of the prostate than in the ventral portion.[2] There is little smooth muscle and less stromal tissue in the canine prostate than in the human prostate. The human prostate also can be separated into three distinct histologic zones (peripheral, transitional, and central); these zones are not evident in dogs.[3]

The primary blood suppy arises via the internal pudental artery as the paired prostatic arteries. The right and left lobes of the prostate are vascularized independently, with the prostatic artery on each side separating into cranial, middle, and caudal arteries. In general, vascularity is poor on the ventral prostate surface compared to the rest of the gland.[4]

Clinical prostatitis

In men, prostate infection or inflammation is classified as acute infectious prostatitis, chronic infectious prostatitis, non-infectious chronic pelvic pain syndrome, or asymptomatic prostate inflammation.[5,6] In dogs, acute and chronic prostatitis are recognized disease entities, with prostatic abscessation generally considered a companion to either. Chronic prostatitis without abscessation will be the focus of this discussion.

Chronic prostatis occurs in the presence of some other prostate disease. The normal canine prostate is protected from ascending infection by anatomic, functional, and immunologic barriers, including secretion of prostatic fluid and urine flow, urethral peristalsis, formation of a urethral high pressure zone at the area of the prostate, and antibacterial properties of prostatic fluid.[7,8] Likelihood of infection is increased by any process that increases the number of bacteria in the periprostatic urethra or urinary bladder, or that compromises local or systemic immune response.[9] Specific examples include urolithiasis, urethral neoplasia, urinary tract infection, squamous metaplasia of the prostate due to hyperestrogenism with subsequent decrease in prostate secretion, and concurrent prostate diseases such as benign prostatic hypertrophy (BPH) or neoplasia.[7]

The class of organism most often associated with chronic prostatitis in dogs is the Gram negative Enterobacteriaciae, including *E. coli*, *Klebsiella sp.*, and *Proteus sp.* Other bacterial organisms reported include *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Brucella canis*.[10-12] Mycoplasma has been reported as a cause of chronic prostatitis,[11] as has blastomycosis.[13] Bacterial organisms implicated in human prostatitis are the same as those in dogs. A complicating factor in human medicine, which may occur in veterinary medicine, is formation of biofilms, a matrix of bacteria and polysaccharide that permits creation of antibiotic-resistant microcolonies within tissue.[14]

Clinical signs are mild, with disease often inapparent. Reported clinical presentations include infertility, poor semen quality, and signs of associated prostate disease such as dripping of bloody fluid from the penis in dogs with concurrent BPH.[12]

Diagnosis is via rectal palpation, imaging, and collection of samples of prostatic tissue or fluid for cytology or culture. Rectal palpation is not an accurate diagnostic test; in one study of 500 dogs, rectal palpation permitted correct identification of prostate disease in only 53% of cases.[15] Rectal palpation can be used to judge prostate size and to assess for a pain response, which usually will be absent in dogs with chronic disease. There are no pathognomic changes visible by ultrasound that permit definitive diagnosis by imaging alone.[16] Most often the

infected prostate is described as locally to diffusely hyperechoic.[17] Ultrasound may be best used to guide sample collection; ultrasound-guided fine-needle aspirate has been reported to be 75% accurate in identifying prostatitis.[18,19]

Culture of prostatic fluid or tissue is required for definitive diagnosis of prostatitis. Culture of ejaculated prostatic fluid is sensitive but not specific as it may be contaminated with organisms from the urinary tract.[20] Results from culture of prostatic fluid correlated well with culture of prostatic tissue in only 80% of instances in one study.[21] Similarly, presence of inflammatory cells in ejaculated prostatic fluid is not well correlated with culture results.[21,22] For these reasons, cytology and culture of prostatic tissue is preferred when possible. The only reported side-effect of fine-needle aspirate of the prostate is transient hematuria.[23]

Treatment involves management of concurrent prostatic or urinary tract disease and antibiotic therapy, as described in detail below. It has been well demonstrated that castration hastens resolution of chronic prostatitis in dogs without prostatic neoplasia as an underlying cause of disease.[11,24] Medical treatment of underlying BPH with finasteride may hasten resolution of disease; the author is unaware of studies evaluating effect of finasteride therapy as a component of treatment for prostatitis in dogs.

Antibiotic pharmacokinetics relative to the prostate

Antibiotics may be characterized by their absorption, distribution, metabolism, and excretion. Factors that affect these processes include lipid solubility, molecular weight, and ionization of the drug molecule, and degree to which the drug is bound to protein in circulation. For antibiotics to penetrate tissue, they must be able to pass through the lipid-rich cell membrane, either by active transfer or diffusion. Since most antibiotics move into cells passively, lipid solubility and size of the molecule are crucial determinants of ability to penetrate tissue. Environmental pH alters ionization of the cell, which also affect permeability as ionized drug cannot pass through the lipid bilayer. Finally, drug that is bound to protein in serum is unavailable compared to drug that is dissolved and can freely cross cell membranes.[25,26]

Ionization and trapping of drugs due to altered pH has long been upheld as a component of the decision regarding which antibiotic to choose for treatment of chronic prostatitis. The premise is that because charged (ionized) molecules cannot cross the cell membrane and because there may be a pH gradient from serum into prostatic tissue as disease develops within the prostate, uncharged molecules will equilibrate across that membrane but charged molecules will get trapped on one side or the other. If you could trap molecules within the tissue, you'd get a higher concentration of drug on that side (uncharged plus charged portions of the total drug present).[26] Knowledge of the pKa, or ionization constant, would permit one to choose an appropriate antibiotic based on pH of prostatic fluid. The value of this premise is decreased in small animal practice as it has been demonstrated that pH of canine prostatic fluid does not significantly change in the presence of prostate disease,[21] and because of the availability of antibiotics that can ionize at either acicid or alkaline pH.

Fluoroquinolones

Fluoroquinolones are the preferred class of antibiotics for treatment of chronic infectious prostatitis in humans. The fluoroquinolones are bactericidal via inhibition of DNA gyrase, which is necessary for DNA replication and repair.[25] They are well distributed throughout the body because they are lipid soluble and are amphoteric, with both acidic and alkaline ionization constants.[27-29] Fluoroquinolones also move well into biofilms, lessening chance of persistent or recurrent infection.[27] Products available for oral use in dogs include enrofloxacin (Baytril®; Bayer Animal Health, Shawnee Mission, KS, USA), ciprofloxacin (Cipro®; Schering-Plough, Kenilworth, NJ, USA), marbofloxacin (Zeniquin®; Pfizer Animal Health, New York, NY, USA), orbifloxacin (Orbax®; Intervet/Schering-Plough Animal Health; Summit, NJ, USA), and difloxacin (Dicural®; Fort Dodge Animal Health, Ft. Dodge, IA, USA). Of these compounds, enrofloxacin shows superior movement into canine prostatic tissue, followed by ciprofloxacin and marbofloxacin.[28,30-32]

Macrolides

The macrolide antibiotics are bactericidal or bacteriostatic, depending on bacterial species.[25] Erythromycin is lipid-soluble but is quickly broken down after oral administration and is highly protein bound.[27] Azithromycin concentrates in phagocytes and so is carried into tissue, from which it is slowly released, permitting less frequent dosing.[27,33-35] It has been demonstrated to penetrate the prostate well in humans.[27] There are no veterinary formulations of azithromycin available as of this writing.

Chloramphenicol

Chloramphenicol is bactericidal or bacteriostatic, depending on bacterial species.[25] It is very lipid-soluble and not highly protein bound.[27] Because tissue concentrations generally are well below those in serum, high doses must be used, raising concerns about toxic effects, including myelosuppression, with long-term use.[7]

Trimethoprim-Sulfas

Trimethoprim-sulfa combinations are bacteriostatic as they inhibit DNA replication.[25] These drugs are well absorbed orally and penetrate prostate tissue well.[27] Concerns of long-term use include keratoconjunctivitis sicca,

anemia, and acute neutrophilic hepatitis.[27] The approved veterinary product for dogs is trimethoprim-sulfadiazine (Tribrissen®; Intervet/Schering-Plough).

Other antibiotics

Penicillins and cephalosporins are poorly lipid-soluble and do not penetrate the chronically infected prostate.[27] Aminoglycosides (amikacin, gentamycin) show variability tissue penetration and potential toxicity with long-term use.[27,36] Doxycycline penetrates prostate tissue better than tetracycline but is not commonly used.[27] Metronidazole (Flagyl®; Pfizer, Inc, New York, NY, USA) may be useful if anaerobic infection is a component of disease; it is a lipid-soluble antibiotics that is well absorbed orally and is not highly protein bound.[27] Neurologic signs and gastrointestinal signs may be seen as side-effects.[27]

Conclusion

The primary factor governing antibiotic choice must be the result of culture and sensititivity testing, preferably of prostatic tissue. The chosen antibiotic should be administered for 4 to 6 weeks, with rechecks of prostatic fluid or tissue culture at 7 days, one month and 6 months after completion of therapy.[29,37] Antibiotic resistance may be a growing concern with wide use of fluoroquinolone antibiotics;[38] for this reason an effort should be made to decrease risk of recurrence. Concurrent disease of the urinary tract or prostate must be addressed, including a recommendation for castration in dogs with BPH and recurrent or persistent prostatitis.

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Neoplasia of the reproductive tract of the male dog

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Abstract

A brief review of tumors of the male reproductive tract is provided. Diagnosis is straightforward in most cases, with resolution of disease easily achieved. The exception to the latter is prostatic neoplasia, a high morbidity, high mortality form of cancer to which we may predispose dogs by castration.

Keywords: Neoplasia, penis, prostate, testes, transmissible venereal tumor

Introduction

Neoplasia of the male canine reproductive tract is uncommon and often easily diagnosed. The most common reproductive tract tumor of male dogs worldwide is testicular neoplasia, with transmissible venereal tumor also very common. The most devastating cancer of the reproductive tract of male dogs is prostatic neoplasia. Less common tumors include those of the soft tissues of the penis and prepuce, those of the os penis, and those of the scrotal skin and mammary tissues. Excellent published reviews of this topic exist.[1,2] This manuscript will provide brief reviews of cancer biology and updated information.

Testicular tumors

The three most common tumor types are seminomas, Leydig (interstitial) cell tumors, and Sertoli cell tumors. In humans, testicular neoplasia is common in young men and is aggressively malignant. In dogs, testicular neoplasia occurs in older individuals and is malignant in 10% of cases at most. It is not uncommon for there to be more than one tumor type in a given testes or between testes concurrently.[3,4]

Incidence of testicular neoplasia is higher in retained than in descended testes. There is no research documenting increased risk of neoplasia in the descended testis of unilateral cryptorchids.[5] Controversy exists regarding whether or not the predisposition to neoplasia in retained testes remains if the cryptorchid testis is surgically pulled down and tacked into the scrotum. In human medicine, studies suggest increased incidence in tumor incidence if orchiopexy is delayed until puberty or beyond.[5,6] This may suggest that medical therapies to induce testicular descent are beneficial in prepubertal dogs, both by potentially decreasing tumor incidence and making castration of those dogs easier.

Dogs with testicular neoplasia may present with enlargement of one or both testes. If neoplasia is unilateral, oftentimes there will be atrophy of the contralateral testis due to increased intrascrotal temperature and possibly to hormone secretion by the affected testis. Components of an associated paraneoplastic syndrome, most commonly associated with estrogen secretion, include gynecomastia, attraction of male dogs, and bilaterally symmetrical alopecia. Malignant tumors mestastasize locally and to the regional lymph nodes and lungs, with occasional reports of hypertrophic osteoarthropathy.[7] A fair proportion of dogs (28.3% in one study) have inapparent testicular neoplasia; these dogs may present for testicular atrophy, poor semen quality, or infertility.[3]

Diagnosis is by inspection. For dogs with no overt change in testicular size or consistency, testicular ultrasound is the preferred diagnostic technique. Fine-needle aspirate (FNA) of the testes also may be used to identify abnormal cells. The dog is sedated and the scrotal skin cleaned. A 20 ga needle is attached to a 12 cc or larger syringe. The needle is introduced on or just lateral to the midline and redirected several times, with suction at each location. Negative pressure is released and the needle withdrawn. The sample is expelled onto a glass slide and submitted to a cytologist for interpretation.[8-10]

Treatment is surgical. Bilateral castration is recommended. Caution during surgery is recommended; there are reports of neoplastic tissue (n = 11 Sertoli cell tumors, n = 1 Leydig cell tumor) arising in the spermatic cord, at the incision site, on the scrotal skin, and in the inguinal canal, presumably due to transplantation of cells during castration.[11] In valuable breeding animals, one may consider removing only the affected testis but the owners must be cautioned that spermatogenesis may be altered by inapparent changes in the remaining testis.

In humans, incidence of testicular neoplasia is increasing. This same trend appears to be true in veterinary medicine, with incidence of testicular tumors increased in one study from that described in 1962.[12] The primary theory explaining this in human medicine is increase in exposure to environmental toxins.

Transmissible venereal tumor

Transmissible venereal tumor (TVT) is a cellular transplant containing an average of 59 chromosomes, rather than a transformed canine cell, which would contain 78 chromosomes. Analysis of TVTs from five continents showed that all are genetically similar, suggesting that all arose from a single mutation. That mutation is believed to have arisen 250 to 2500 years ago in a wolf or east-Asian breed of dog.[13]

Incidence of TVT in one study was 5.4%; this incidence varies greatly by region.[4] TVT is most common in young, sexually intact, free-roaming dogs, and so is primarily a problem in sub-tropical and tropical areas of the world.

Most TVTs invade only locally and so usually are not considered malignant. The tumor is a meaty and irregular, and is easily ulcerated. Lesions may be noted on the mouth and nose; this is from transplantation of cells by licking of the genitalia. Occasionally tumor will spread to regional lymph nodes and the internal organs.[14] Metastasis is more common in very young animals and immunosuppressed animals.[15]

Diagnosis is by cytology. TVT is easily identified as round cells with abundant cytoplasm and an eccentrically placed round to oval nucleus.

Chemotherapy with vincristine is the recommended treatment. Surgical debulking may be required in some cases. Vincristine treatment has been demonstrated to cause resolution of clinical signs with minimal side-effects within 4 treatments in most dogs, and to decrease semen quality in breeding dogs for only up to 15 days after treatment is completed.[16,17] Treatment failure is more likely if tumors are large, the affected animal is old or immunosuppressed, and if the treatment is performed during hot or rainy months.[18]

Prostatic neoplasia

The most common prostatic neoplasm reported is adenocarcinoma. It is considered to have metastasized by the time of diagnosis in the majority of cases. It is a disorder of older male dogs and may occur in either intact or castrated dogs. It is the only prostatic disorder commonly seen in castrated dogs. Overall incidence of prostatic neoplasia is reported as 0.4 to 0.7%.[19,20] It is a high mortality disorder; in one survey of 72 affected dogs, 58 were euthanized at the time of diagnosis and mean survival for those who survived more than one week from diagnosis was 30 days.[21]

In humans, prostatic neoplasia is hormone-dependent. That is not true in dogs. In fact, castration, and subsequent removal of testosterone, appears to predispose dogs to prostatic neoplasia, with increased risk of 2.8 to 4.3 times reported.[22,23] Castrated dogs tend to develop more poorly differentiated tumors than intact dogs, suggesting that testosterone has a protective effect.[24] Number of androgen receptors is decreased in the prostates of dogs with neoplasia compared to dogs with normal prostates or benign prostatic hypertrophy, again suggesting loss of a protective effect of testosterone.[25] In one survey of 56 affected dogs, time from castration to onset was variable and there was no difference in mean age at time of diagnosis between intact and castrated dogs, suggesting that castration does not favor tumor initiation but may favor tumor progression.[20]

It has been shown in dogs that castration leads to an increase in number of receptors for endothelin on the prostate; endothelin increases mitogenic responses and uncontrolled cell growth.[26] Endothelin also supports osteoblastic function and stimulates bony growth at areas of metastasis from prostatic neoplasia in dogs.[27]

Dogs may present with signs of prostate disease, such as dripping of bloody fluid from the penis unassociated with urination, hematuria, and passage of ribbon-shaped stools. Commonly, dogs also present with stranguria and signs referable to sites of metastasis including stiff gait or ataxia, coughing, and cachexia.

Because prognosis is grave, definitive diagnostics are required. Definitive diagnosis requires collection of a sample directly from the prostate, either as FNA or biopsy. Ultrasound guidance is recommended with either technique. For FNA, sedation usually is not required. A 20 ga needle is attached to a 12 cc syringe. The needle is passed into the prostatic parenchyma and negative pressure applied. Pressure is released and the needle withdrawn. The sample is expelled onto a glass slide and submitted to a cytologist for interpretation. For biopsy, sedation is required. Prepare the area over the prostate as for sterile surgery and place a sterile sleeve over the ultrasound probe. Viewing the prostate by ultrasound, trigger the biopsy instrument to see it pass within the prostatic parenchyma. Withdraw the biopsy instrument and retrieve the sample; make sure you have an adequate sample before reversing sedation. Samples should be submitted to a pathologist for interpretation. A diagnostic sample is more commonly retrieved by biopsy than by FNA. Side-effects include hematuria and hemospermia.[28,29] In humans, seeding of the abdomen with tumor cells and subsequent growth of transplanted cells is reported to occur in 0.009% of cases after these techniques.[30] This is less of a concern in dogs, who rarely live long enough after diagnosis for significant secondary tumor development to occur.

Treatment is palliative. Most dogs are treated with anti-inflammatory medications (meloxicam, piroxicam) and antibiotics to control secondary infection. Prostatectomy rarely is performed because it cannot cure disease and often causes urinary or fecal incontinence. There are reports of transurethral resection of the prostate using electrocautery, with or without associated radiation and chemotherapy,[31] and subcapsular prostatectomy using a laser.[32] Some of the dogs in these studies showed a resolution of clinical signs and had longer mean survival times than is commonly reported but other dogs in those studies did not respond to treatment or died from complications of therapy. That, coupled with the technical skill required to perform this work, makes them unsuitable alternatives for most veterinarians and their patients.

Tumors of the penis, prepuce, scrotum, and mammary tissue

Tumors of the soft tissue of the penis and of the os penis are described in the literature. Tumors of the soft tissue of the penis often are associated with preputial extension and, along with tumors of the scrotum, are the

common skin tumors seen elsewhere including papillomas, squamous cell carcinoma, lymphosarcoma, and mast cell tumors.[33,34] Tumors of the os penis are less common and may be either benign or malignant. Stranguria is a common presenting complaint with tumors of the os penis, which may be confused with fracture of the os penis.[34-38] Mammary neoplasia rarely is reported in male dogs; one institution reported that male dogs at that institution were 62 times less likely to develop mammary neoplasia than female dogs in that population.[39] Mammary tumors usually are benign and easily surgically removed.

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Endometritis in dogs – current knowledge and future considerations

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Abstract

Canine endometritis, as an entity separate from cystic endometrial hyperplasia, is a poorly understood process, yet may be responsible for infertility. Inflammatory infiltrates of the endometrium in the absence of proliferative changes are the hallmark of this disease. The ascension of bacteria from the vagina through the cervix is theorized to lead to a chronic, low-grade endometritis, which does not produce overt clinical disease, such as that seen with pyometra. *Escherichia coli* is the organism most commonly implicated in pyometra, and is likely the most common organism involved with endometritis. Various uropathogenic virulence factors (UVFs) have been identified from uterine isolates, many of which share common UVFs from isolates producing urinary tract disease in dogs and humans. Of these, P fimbria is thought to be crucial for initial bacterial adherence to the luminal epithelium. Reduction in Mucin-1 expression and immunolocalization in endometrial epithelial cells in early diestrus may be involved with producing a permissive state for bacterial adhesion, allowing colonization. Samples that may aid in the diagnosis of endometritis include vaginal or uterine cultures, uterine biopsy, and possibly endometrial cytology and ultrasonography. Treatment of endometritis is focused on elimination of infection through the use of antimicrobial agents, and physical clearance of the uterus through the use of prostaglandins, dopamine agonists, and progesterone-receptor antagonists.

Keywords: Dog, infertility, endometritis, diagnosis, treatment

Introduction

Although many clinicians might empirically agree that inflammation of the endometrium may contribute to a reduction in fertility, direct evidence of a causal effect of endometritis on reducing fertility is lacking. Subfertility and infertility due to cystic endometrial hyperplasia is more generally agreed upon; however, whether the reduction in fertility is due to the proliferative response of the endometrium or the inflammatory component of the process is unknown.

Histopathology of the canine endometrium

Endometrial hyperplasia, with or without cystic changes is considered a post-estrual luteal phase disease. Age and nulliparity are considered risk factors for development of CEH.[1,2] Two main hypotheses exist on the pathogenesis of CEH and subsequent development of pyometra. The classical description involves the development of endometrial hyperplasia which subsequently leads to an inflammatory reaction. The accumulation of secretions from the hyperplastic endometrium supports the growth and proliferation of bacteria which ascend through the cervix. Establishment of bacterial infection leads to the accumulation of exudate (pyometra). An alternative hypothesis centers on the premise that chronic low-grade uterine infection drives the endometrial proliferative response either by bacterial toxins or inflammatory mediators.[3] It has also been suggested that endometritis-pyometra can occur independent of endometrial hyperplasia; unfortunately the age distribution of these cases were not defined.[4] A variety of stimuli have been used to experimentally reproduce endometrial proliferation. These have ranged from china balls,[5] suture,[6-8] and bacteria,[9-13] among others. The proliferative changes observed have been well-characterized, and are not the focus of this discussion. For detailed information on CEH, the reader is directed to several excellent reviews on the topic.[1,3,4,14]

Endometritis is also a common finding, and was recently determined to be the most common diagnosis (94 cases) in a survey of 366 canine endometrial biopsies. Hyperplasia was the second most common diagnosis (86 cases).[15] It is most common to find a plasmacytic infiltrate with subclinical endometritis[14] or with CEH with plasmacytic infiltration and little intraluminal fluid accumulation,[1] progressing to neutrophilic plasmacytic infiltrates in cases of CEH and significant fluid accumulation (pyometra).[1]

Microflora of the reproductive tract

Debate exists on whether vaginal cultures are useful for determining the presence of intrauterine infection. Although most prepuberal and postpuberal bitches were found to have positive vaginal cultures, the majority of uterine cultures were found to be negative.[16] Similarly, all uterine swabs collected from late diestrus, progestin-supplemented bitches were sterile,[17] although concurrent sampling of the vagina was not performed. The predominate isolate from infected uteri is *Escherichia coli*,[1,18-22] with reported incidences of 73%,[1] 79.4%,[21] and 85%.[22] Although *E. coli* in pure culture was the most common isolate from bitches with pyometra, mixed cultures were the most common finding in 'infertile' bitches, leading to the conclusion that vaginal cultures have low diagnostic value.[20] Conversely, other studies found the uterus not to be a sterile environment,[23,24] and that vaginal isolates reflected those of the uterus.[23] Bacteria were consistently recovered from the uterus during proestrus and

estrus, and post-mortem uterine isolates always reflected those of the cervix and vagina.[24] The most common uterine isolates were *E. coli*, *Haemophilus* spp., α-hemolytic streptococci, *Corynebacterium* spp., *Streptococcus canis*, *Alcaligenes faecalis*, *Bacteroides* spp., *Pasteurella* spp., and *Proteus mirabilis.*[24] A method to transcervically collect uterine secretions that is guarded from vaginal secretions would enhance the ability of clinicians to accurately diagnose the presence of bacteria in the uterus.

Several authors have investigated virulence factors of *E. coli* isolates from cases of pyometra. Early characterizations focused on the presence of the O-[18] and K-antigen[21,22] Uropathogenic *E. coli* strains, which are responsible for urinary tract infections in dogs and cats, may originate from the intestinal tract, and possess a cluster of virulence-related genes encoding for specific O-antigens, type 1 fimbriae, P fimbriae, S fimbriae, α-hemolysin, cytotoxic necrotizing factor 1, and aerobactin (iron-sequestering system).[25-27] These strains are not canine-specific, and it has been suggested that the dog may serve as a source of uropathogenic *E. coli* for human urinary tract infections (UTI).[28,29] Biochemical fingerprinting of *E. coli* isolates from pyometra and UTI suggest that these isolates originate from the fecal flora, and the same clone of *E. coli* is present in cases with concurrent UTI and pyometra.30 Similarly, DNA-profiles of *E. coli* isolates from the urinary bladder and uterus of bitches affected simultaneously with UTI and pyometra were 100% identical, and that all colonies from a site were identical, despite macroscopic morphologic differences.[31] The *papGIII* allele, the most frequent allele encoding for P fimbriae in canine and human uropathogenic *E. coli* isolates,[29] had a significantly higher prevalence in *E. coli* isolates from pyometra, and the proportion of strains from pyometra possessing more than three uropathogenic factors was greater than that of fecal strains.[32] The presence of P fimbriae is thought to be crucial for bacterial adherence to epithelial cells of the urinary tract. The PapGIII adhesion binds to Galα1-4 Galβ-containing glycolipid receptor and its coreceptor TLR4 present on urinary epithelial cells.[33] The presence of these receptors has been confirmed for canine urinary epithelium,[34] but has not been investigated in canine endometrium. Other virulence genes that have been associated with uropathogenicity and were present in high proportions of isolates from pyometra isolates include *fim* (Type I fimbriae) and *sfa* (S fimbriae), although the differences were not statistically significant.[32] Although Type I fimbriae are present on many isolates from human UTIs, the correlation with pathogenicity is considered low.[35] *Escherichia coli* bearing S fimbriae bind to human renal proximal tubular cells,[36] but only 27.4% of strains were positive for *sfa* compared to 97.5% of strains carrying *fimH* (Type I fimbriae).[37]

These findings have led researchers to the conclusion that the pathogenesis of both UTI and pyometra involve ascension of intestinal strains of *E. coli* into the lower urinary tract, cranial vagina, and uterus.[28,30,32] Only a few investigators have attempted to induce infection by inoculation with *E. coli*. In a series of investigations using an *E. coli* isolate from a clinical case of pyometra, Nomura et al. inoculated the uterus of dogs in either pro-estrus/estrus, diestrus, post-partum, or anestrus with or without cervical ligation.[9,11,12] When examined 12 d post-inoculation with cervical ligation, the incidence of pyometra in proestrus/estrus, diestrus, post-partum, and anestrus was 100%, 100%, 80%, and 28%, respectively.[9] When examined 12 d post-inoculation without cervical ligation, the incidence of pyometra in pro-estrus/estrus, diestrus, post-partum, and anestrus was 25%, 89.9%, 70.6%, and 50.6%, respectively. More recently, 5×10^7 CFU of an *E. coli* (O2:H:K) isolated from a clinical case of pyometra was inoculated in the uterus of intact bitches at either post-LH day 1-10, 11-20, 21,30, 31-40, 41-50, or 51-60; the incidence of pyometra induced was 16.7%, 90.9%, 78.9%, 62.5%, 40.0%, and 0%, respectively. Bitches with induced pyometra were either treated with dinoprost tromethamine and enrofloxacin, or were allowed to spontaneously recover. There was no difference in pregnancy rates between treated and non-treated bitches on the subsequent estrus, and recurrence of pyometra did not occur.[13] While this model did induce pyometra in the strict sense of the definition (the presence of pus in the uterus during the luteal phase), the subsequent fertility and lack of recurrence do not fit the typical clinical scenario of bitches with spontaneous pyometra. This model appears to more closely approximate endometritis than pyometra. Subsequently, inoculation of the uterus with an E. coli strain possessing five UVFs induced CEH/pyometra in diestrus-simulated ovariectomized bitches, while inoculation into the vagina failed to establish uterine infection or endometrial changes.[10] Differences in these two studies lie in the status of the bitch and potential difference in the pathogenicity of the *E. coli* strain.

Host-pathogen interactions

Limited investigation on the host-response to intrauterine infections exists, but a few noteworthy studies shed some light on mechanisms by which bacterial are able to colonize the endometrium. The proliferative response of peripheral blood monocytes (PBMCs) to a clone of *E. coli* isolated from the uterus from a dog with pyometra was significantly decreased at day 10 of diestrus compared to proestrus, estrus, day 30 of diestrus, or anestrus.[38] Similarly, the addition of progesterone or 5α-dihydroprogesterone to PBMCs collected from anestrous bitches significantly reduced the response to *E. coli* compared to PBMCs supplemented with estradiol 17-β, 17α-hydroxyprogesterone, or pregnenolone; and progesterone reduced the expression of IFNγ by PBMCs compared to estradiol.[38]

Recently lactoferrin, an antimicrobial and immunomodulator member of the transferrin gene family which is expressed by epithelial cells and neutrophil granules, has been identified in the equine and canine endometrium.[39,40] Lactoferrin's antibacterial property lies within its ability to sequester free iron, thereby inhibiting bacterial growth. In the mare, lactoferrin expression was upregulated during early estrus, protein staining was uninfluenced by cycle and was most intense in the glandular epithelium, and expression of lactoferrin was only increased in mares with delayed physical clearance during early estrus,[39] which might represent a response to inflammation. The pattern of lactoferrin expression has also been described in the bitch, where expression increased from proestrus to estrus, then significantly decreased from estrus to day 10 of diestrus, remaining low at day 35 of diestrus and anestrus; a similar pattern was observed with immunohistochemical staining for lactoferrin,[40] leading the investigators to conclude that estrogen was involved with the regulation of lactoferrin expression. Although reduced lactoferrin expression during diestrus would be a plausible explanation of reduced microbial defenses and increased susceptibility to infection, lactoferrin expression was increased in bitches with pyometra,[40] similar to what was observed in mares with delayed uterine clearance and post-mating induced endometritis.[39] Increased lactoferrin expression in both instances may due to an influx of neutrophils.[41] While intriguing from a perspective of host-pathogen interactions, unless diminished response in lactoferrin expression and production were observed in bitches suffering from pyometra, reduced lactoferrin activity is unlikely to be responsible for increased susceptibility to infection.

Mucin-1 (Muc1) is an important component of the epithelial cell glycocalyx, functioning as an anti-adhesive molecule; loss of Muc1 expression is considered an integral step in allowing adhesion between the trophoblast and the luminal epithelium.[42] In normal, cyclic bitches, Muc1 expression and localization was significantly decreased at day 10 of diestrus and in bitches with pyometra compared to proestrus, estrus, day 35, or anestrus. Additionally, Muc1 expression and adherence of E. coli to endometrium was inversely correlated.[43] Clearly, further research is needed in the area of host-pathogen interactions of the canine uterus.

Diagnosis of endometritis

If bacteria such as *E. coli* can serve as a stimulus for endometrial proliferation, and hence cystic endometrial hyperplasia; then early diagnosis and appropriate therapy might lead to prolongation of the fertile lifespan of some bitches. For additional detail on specifics for collecting reproductive tract tissues, the reader is directed to an excellent previous review on this topic.[44]

Cultures – The most common method used clinically is guarded culture of the anterior vagina during proestrus. As discussed previously, these may or may not reflect a potential uterine pathogen, but in cases in which significant intrauterine infection is suspected, it is the opinion of this author and others,[44] that this method should yield a satisfactory sample. It is also possible to collect intrauterine secretions following transcervical catheterization with a 4 to 7 Fr catheter through a rigid cystoscope or endoscope in the standing bitch.[45] Unfortunately this sample would also suffer from contamination by vaginal fluids. Hysteroscopy in the anesthetized bitch may lessen the contamination, but caused petechia or ecchymoses in 50% of cases, and poor visualization in 37.5% of cases.[45] The method providing the most accurate sample of uterine secretions would be that obtained during hysterotomy. In most instances this sample would be obtained concurrent to uterine biopsy.[46]

Cytology – Endometrial cytology is a commonly used diagnostic tool for the diagnosis of equine endometritis; however, it is not routinely used for evaluation of canine cases. Watts et al.[47] described the endometrial cytology of the normal bitch in samples collected by transcervical catheterization or at post-mortem.[45] Endometrial cells were present at all stages, and exhibited degenerative changes during late diestrus, anestrus, and postpartum. During proestrus and estrus, healthy endometrial cells, neutrophils and bacteria were commonly observed. During diestrus and early pregnancy, healthy endometrial cells and neutrophils were most common. During late diestrus and anestrus, evidence of endometrial cell degeneration was observed and lymphocytes and macrophages were the most common leukocyte present. To the authors knowledge there are no published reports regarding changes in endometrial cytology with uterine pathology such as endometritis or cystic endometrial hyperplasia.

Uterine biopsy – Biopsy of the endometrium is maximally invasive, yet provides the most accurate sample for the diagnosis and prognosis for endometritis or other uterine pathology. Samples can be obtained by either laparotomy,[46] or by a transcervical approach;[48] however, the later technique only provided a diagnostic sample 31% of the time and was associated with hematomucometra in 44% of cases.

Ultrasonography – Ultrasonography is commonly used to diagnose cystic endometrial hyperplasia with or without pyometra. Its usefulness for the diagnosis of endometritis has not been correlated with other diagnostic techniques.

Treatment of endometritis

From the preceding discussion, it is apparent that arriving at an accurate diagnosis of endometritis may be difficult. No controlled studies have been done on therapeutic regimes for the treatment of endometritis. Therefore, treatment options are based on what has been recommended for medical management of pyometra, which is focused

on eliminating bacterial infection, if present, and stimulating physical clearance of the uterus. The former is achieved by appropriate antimicrobial agents, the latter by terminating the luteal phase through the use of prostaglandins, dopamine agonists, or progesterone-receptor antagonists, and by stimulation of myometrial contractions through the use of prostaglandins. Prior to initiating medical therapy, detailed owner counseling regarding the intended use and breeding value of the bitch should occur. Ovariohysterectomy should be recommended for bitches without significant reproductive value.

Antimicrobial agents – When at all possible, antimicrobial agents should be chosen based on results of culture identification and sensitivity patterns. The injudicious use of antibiotics has the potential to select for strains of bacteria with greater antibiotic resistance patterns. To the author's knowledge multi-resistant *Staphylococcus aureus* (MRSA) has not been isolated from a canine case of endometritis or pyometra. This would be a dire situation indeed. Recently, MRSA has been recovered from the uterus of mares with extensive history of intrauterine antibiotic therapy.[49] Systemic administration in the bitch is the most commonly used route of delivery. Although transcervical delivery of antibiotics into the lumen is possible, it is doubtful that uniform distribution of drug to the entire endometrial surface would occur. Additionally, daily treatment would be required; repeated catheterization adding considerably to the cost of treatment. Systemic administration allows for longer treatment regimes (10-14 d), with good penetration of the endometrium.

Prostaglandin – Prostaglandin has been used for many years for the treatment of pyometra.[50,51] Typical doses of $PGF_{2\alpha}$ (dinoprost tromethamine) range from 100 to 500 µg/kg, SQ, one to three times daily, and are considered luteolytic. Lower doses (10 to 50 µg/kg, up to three to five times daily) can also be used to achieve uterine evacuation.[52] The use of prostaglandin in the dog is extra-label; therefore, informed client consent is recommended. Transient (20 to 30 min) side effects of salivation, defecation, urination, and emesis are not uncommon at the higher doses, but are drastically reduced when lower doses are used.

Dopamine agonists – Inhibitors of prolactin will aid in the rapid reduction in progesterone concentrations, and are frequently combined with low-dose prostaglandin protocols for the treatment of induced abortion.[53,54] Bromocriptine (25 µg/kg, PO, q8 to 12 h) or cabergoline (5 µg/kg, PO, q24 h), in combination with prostaglandins, can lead to luteolysis with 24 h.

Progesterone-receptor antagonists – Although not available in the United States, aglepristone has been used for pregnancy termination,[55] and for treatment of pyometra alone[56] or combination with cloprostenol for pyometra.[57] In a recent study the overall success rate for treatment of pyometra (35 open, 17 closed) with cloprostenol (1 µg/kg) and agelpristone (10 mg/kg, daily) was 84.4% compared to 60% for aglepristone alone; the recurrence rate at 12 and 24 mo was 13% and 19% respectively.[58]

Discussion

It would seem that there is rather compelling evidence to suggest that subclinical endometritis may precede the development of clinically evident CEH and pyometra. The difficulty lies in the ability to render a diagnosis prior to development of proliferative changes in the endometrium. Clearly further research is needed to prove a causal relationship between these two entities. Comparison of *E. coli* strains possessing or lacking UVFs in a model would be a useful first step in that process. Further investigation of host-defense mechanisms, such as the presence of toll-like receptors on the endometrium, cytokine signaling involved with endometritis and proliferative changes of the endometrium would also be informative. Biofilms have been described in a variety of mucosal systems, and provide a mechanism for pathogen evasion of host recognition and protection from certain treatment modalities. Uropathogenic E. coli producing biofilms are implicated in chronic urinary tract infections in people,[59] and it has been suggested that some cases of E. coli endometritis produce biofilm.[60] From a diagnostic standpoint, a guarded system for transcervical collection of uterine secretions in the standing bitch would benefit not only research endeavors, but collection of diagnostic samples from clinical cases. Such information is needed to render an accurate diagnosis, and to progress towards effective treatment strategies.

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Table 1
Function, classification, and distribution of virulence factors of *Escherichia coli* isolates from various species.

Factor	Serogroup or Genes	Site	Species
O Antigens thermostabile; agglutinating; immunogenic O-specific polysaccharide of the cell wall lipopolysaccharide; possessed by all smooth forms of *E. coli*	O1, O2, O4, O6, O25	urinary tract [28]	dog [26,28] human [28]
	O1, O2, O4, O6, O7, O8, O22, O23, O25, O32, O45, O75, O83, O88 O147	uterus [18,21]	dog [18,21]
type I fimbriae present on most E. coli; bind cell-bound and secreted mannosylated glycoproteins, Tamm Horsfall protein, and uroplakins of bladder epitheliam; presumed to be able to bind to endometrium	*fim (pil)*	urinary bladder [33] feces [25, 32] uterus [32]	dog [25, 32] mouse [33]
P fimbriae mediate attachment to Galα1→4Galβ-containing glycolipid receptor and coreceptor TLR4	*papGIII*	urinary epithelial cells [25,28,33] feces [25,32] uterus [32]	human [28] dog [25,28,32]
S fimbriae bind eukaryotic glycoproteins with a terminal α-sialic acid; bind laminin and plasminogen; may play a role in penetration of *E. coli* across basement membrane	*sfa*	urinary tract [25,28]] uterus[25,32] feces [25,32]	dog [25,28,32] humans [25,28]
α-hemolysin common exotoxin; toxic to a wide variety of mammalian cells	*hlyA*	urinary tract [25,28] uterus [32] feces [25,32	dog [25,28,32] human [25,28]
Cytotoxic Necrotizing Factor 1 belongs to a group of bacterial necrotic substances; associated with outer membrane vesicles; activates Rho GTPases of host cell leading to macropinocytosis by epithelial cells; may function as a means of entry and survival in epithelial cells	*cnf1*	urinary tract [25,28] uterus [32] feces [25,32]	dog [25,28,32] human [25,28]
Aerobactin and other iron-sequestering systems bacterial siderophores (low molecular weight Fe(III)-chelator)	*iuc or aer, fyuA, iutA, iroN*	urinary tract [25,28] uterus [32] feces [25,32]	dog [25,28,32] human [2528]

Neonatology

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Neonatal survival

Average reported neonatal puppy and kitten mortality rates (greatest during the first week of life) vary, ranging from 9-26%.[1] Prudent veterinary intervention in the prenatal, parturient and postpartum periods can increase neonatal survival by controlling or eliminating factors contributing to puppy and kitten morbidity and mortality. Poor prepartum condition of the dam, dystocia, congenital malformations, genetic defects, injury, environmental exposure, malnutrition, parasitism and infectious disease all contribute to neonatal morbidity and mortality.

Neonates that fail to survive to weaning are most commonly stillborn or die within the first three days of life. Factors implicated in perinatal deaths include prematurity, in utero infection with viruses such as canine distemper, canine parvovirus, feline herpes, feline infectious peritonitis, panleukopenia, and feline leukemia virus, as well as anatomic birth defects, birth trauma/dystocia, low birth weight, inadequate nutrition, maternal neglect, and environmental stresses. Optimal husbandry impacts neonatal survival favorably by managing labor and delivery to reduce stillbirths, controlling parasitism and reducing infectious disease, preventing injury and environmental exposure, and optimizing nutrition of the dam and neonates. Proper genetic screening for selection of sires and dams minimizes inherited defects.[1-4] The neonatal period here is defined as the first 4-6 weeks of life.

Keywords: Neonatal, pediatric, physiology, disease

Neonatal physiology

Cardiovascular system

1. The neonate has a low pressure, low volume, low peripheral resistance circulatory system.
2. Higher heart rate, cardiac output, plasma volume and central venous pressure result.
3. Sympathetic innervation of the heart is incompletely developed, response to anticholinergics minimal.
4. Baroreceptor reflexes are present after 4 days of age, prior to that hypotension results from anoxia.[5]

Clinical implications: One of the most important considerations of cardiovascular physiology in the neonate is that in the fetus and during the first 4 days of life bradycardia is not vagally mediated and is indicative of hypoxemia. Although during this time the neonate appears able to resist circulatory failure to a greater extent than the adult animal, it is far more appropriate to supplement oxygen than to administer parasympatholytic agents such as atropine; administration of which will only exacerbate cardiac hypoxemia via increasing oxygen demand in the face of hypoxemia. Additionally, due to incomplete maturity of the autonomic nervous system, the neonate is less able to respond to physiological stresses. Care should be given to maintain the neonate environment such that demands on the cardiovascular system are minimal.

Respiratory system

1. Stimulation of the genital or umbilical region of the neonate induces reflex respiration in the first three days after birth and may be clinically used to stimulate respiration in the immediate post partum period.
2. Normal respiratory rate in the neonate is low, ranging from 10 – 18 breaths per minute during the first week, despite a high metabolic oxygen demand.
3. The mechanisms that control respiratory function (carotid body chemoreceptors) in the newborn develop well before birth but require maturation in the post natal period.
4. The amount of work and pressure that is required by a neonate to maintain tidal breathing is increased as compared to that of the adult due to the high compliance of the chest wall.[5]

Clinical implications. The neonate is very susceptible to the development of hypoxemia and/or jeopardized ventilation and gas exchange due to the immaturity of chemoreceptor responses to hypoxia and chest wall construction. Although there are adaptations present to help compensate for this physiological state, such as an extremely low circulatory failure pressure until four days of age, it is important to recognize that hypoxemia in the neonate may result in life threatening sequelae such as septic shock due to bacterial translocation despite a lack of mucosal lesions. It is vital that the environment be kept free of airway irritants and oxygenation is adequate.

Hematopoietic system

1. At birth the neonate red blood cell exhibits macrocytosis with corpuscle volume decreasing to that of the adult by four weeks of age as fetal red blood cells are replaced by adult red blood cells.
2. The hematocrit of the neonate may be as high as 60 per cent accounting for the red mucous membrane color often noted at birth. By three days of age red blood cell counts have decreased dramatically and

continue to decrease for approximately three weeks. Adult levels for red blood cell count, hemoglobin, and hematocrit are generally not detected in most dogs until six months of age.

3. Neonatal isoerythrolysis is uncommon in the cat and rare in the dog. In the feline, the phenomenon occurs in association with a type A kitten born to a type B queen that has anti-A alloantibodies (agglutinating and hemolytic). White blood cell parameters in the canine and feline neonate are typically consistent with those of their adult counterparts. Lymphocytosis may also be noted in the normal neonate.[5]
   Clinical implications. During the neonatal period, as fetal red blood cells are replaced polychromasia and elevated reticulocyte counts may be noted. Care must be taken to ensure adequate ectoparasite control as iron demands are high; the presence of microcytosis is suggestive of iron deficiency anemia. Extramedullary hematopoeisis is commonly noted in the neonate liver.

Urinary system

1. In the canine, the neonatal kidney is morphologically and functionally immature; nephrogenesis continues for at least two weeks after birth.
2. The canine neonatal kidney is functionally characterized by a low glomerular filtration rate (GFR.), low renal plasma flow (RPF), low filtration fraction (FF), depressed reabsorption of amino acids and phosphate, exaggerated proximal tubule natriuresis and low concentrating ability.
3. Serum creatinine levels and blood urea nitrogen (BUN) concentrations are lower than in the adult animal; typically 0.4 mg/dl and 8 – 10 mg/dl respectively. Serum phosphorous concentrations are elevated; typically 9 mg/dl, due to skeletal growth.
4. At birth arterial pressure is low (50 – 60 mmHg). During renal maturation increased blood pressure and decreased vascular resistance result in an increase in GFR and RPF. In the neonate, renal blood flow is directly correlated with arterial pressure and does not appear to be altered by inhibition of angiotensin until approximately 6 weeks of age.[5]

Clinical implications. A urine sample is easy to obtain from the neonate with gentle stimulation. The immature nature of the kidney alters interpretation of urinalysis. Low urine specific gravity (1.006 – 1.0017) is normal as is detection of protein, glucose and various amino acids due to the immaturity of the proximal tubule. By three weeks of age urine protein and glucose concentrations approach that of the adult dog and urine concentration is expected to compare to that of the adult dog by six to eight weeks of age.

As the neonatal kidney is less able to concentrate or dilute urine, renal blood flow parallels blood pressure, and there is altered sodium excretion by the proximal tubule. Fluid therapy should be administered with care to ensure adequate volume maintenance without over hydration or oncotic loading. Recommended daily fluid rates for the canine neonate range from 60–180 ml/kg/day. Caution must be exercised when administering renally excreted or metabolized antimicrobials (penicillin, ampicillin, cephalosporins, fluroquinolones, and aminoglycosides) to neonates. Generally, ß-Lactam antibiotics (penicillins, cephalosporins) are the antimicrobial drugs of choice, as although the half-life may be prolonged there is a large therapeutic margin. Ceftiofur, for example, administered at 2.5mg/kg SQ q 12hrs, maximum five days is an acceptable antimicrobial choice. Due to altered metabolism of nonsteroidal antiinflammatories the potential for renal toxicity from their use in the neonate is far greater than in the adult animal.[5]

Hepatobiliary system

1. During pregnancy, the maternal placenta supports many functions performed by the liver and biliary system in the adult animal. Prior to birth, the ductus venosis shunts blood through the liver, effectively bypassing the neonatal sinusoid. The canine neonatal liver and biliary system is functionally immature at birth.
2. There is a significant reduction in bile flow in the newborn puppy as compared to the adult dog, and a complete failure of secretin and glucagon to stimulate bile flow at 3-28 days of age. Despite a relative functional cholestasis in the neonate, serum bile acids may be used to detect hepatocirculatory abnormalities in puppies and kittens as young as four weeks. Alkaline phosphatase (ALP) and gamma-glutamyltransferase (GGT) liver enzyme activities are markedly elevated in neonates less than two weeks old and moderately elevated after two weeks of age. Elevations in ALP and GGT enzyme activity have been attributed to placental, colostral, and intestinal activity. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are typically comparable to that of the adult. Alkaline phosphatase can be physiologically elevated during skeletal growth.
3. Postnatal hepatic microsomal enzyme activities at four weeks of age are 85% of that seen in an adult dog. Adult dog levels of microsomal enzyme activity are achieved by four and one-half months of age.[5]
   Clinical implications. At birth, the neonate experiences functional cholestasis with altered liver enzyme serum biochemical profiles. Due to the absence of fully developed microsomal and P_{450} enzyme activity in the neonate until four to five months of age, caution must be exercised when prescribing medication that

requires hepatic metabolism or excretion. The detection of serum increases in GGT and ALP in the newborn may be indicative of colostrum intake and potentially passive transfer.

Gastrointestinal system

1. Dentition eruption in the neonate first occurs at two to three weeks of age. All deciduous teeth should be present by 12-16 weeks of age.
2. At birth, the gastrointestinal tract is sterile and has a neutral gastric pH. It is characterized by a time-dependent increased permeability of the intestinal mucosa which decreases dramatically after ten hours. Normal nursing pup feces are semiformed and tan in color (acholic). GI motility prior to 30-40 days of life is dependent upon pressure gradients rather than electrical intestinal motility.
3. Body temperature is known to have a dramatic effect on gastrointestinal movement in the neonate. At rectal temperatures below 94 °F, ileus develops. As ileus progresses, the willingness to nurse decreases and the necessity for tube feeding puppies increases. Inherent to the tube feeding process is the risk for aspiration and subsequent development of pneumonia.[5]

Clinical implications. Care should be taken to ensure adequate environmental conditions to maintain normal body temperature in neonates to minimize gastrointestinal ileus. Due to altered absorption from increased gastrointestinal permeability and neutral gastric pH in the immediate post natal period, care must be taken if administering oral drug therapy. Diarrhea can result from overeating, and is then complicated by subsequent bacterial overgrowth.

Immune system

1. Five to ten percent of canine neonatal serum antibodies are derived from trans-placental transfer. At birth, the canine neonate is antibody deficient and immunologically incompetent. The acquisition of passive immunity requires adequate ingestion and absorption of colostrum during the first 24 hours of life. Gastrointestinal absorption of colostral antibodies decreases markedly after 12 hours.
2. Providing adequate ingestion of quality colostrum, the puppy is protected by maternally derived immunoglobulins during the neonatal period. Puppies are capable of producing challenge specific antibodies within two weeks and with repeated challenge can produce a secondary immune response at 40 days. However, even by 40 days of age, T cell mitogenesis and differentiation, and phagocytic cell function systems may not be fully mature.[5]

Clinical implications. Incompletely developed immune systems and inadequate thermoregulation during the first days of life make neonates vulnerable to systemic infection (bacterial and viral). Adequate ingestion of colostrum must occur promptly post partum for puppies to acquire passive immunity. The transmission of protective immunity (placental or colostral antibodies) between a bitch and her puppies depends upon the prior existence of adequate serum maternal antibodies. When colostral intake is not possible or is of questionable quality, pooled adult dog serum (20-150 ml/kg SC divided) may be administered to elevate serum immunoglobulin concentrations in the puppy.[5-7]

Neurologic system

1. The neonatal puppy's main activities during the first two weeks of life are sleeping and nursing. The rooting reflex orients the neonate to its source of food, the dam. Vestibular function is present at birth and is important for positioning during nursing. Muscular coordination however is absent. Initial movements are characterized by swimming-like movements of the limbs, while sliding along on the ventral abdomen and thorax. The ability to raise the head is present at birth in puppies and the head may be used initially for righting reflex. An upright posture in puppies cannot be maintained until ten to 14 days.
2. The EEG of the neonatal puppy initially is similar during periods of sleep and waking.
3. At birth the body posture is primarily one of flexion. If suspended by the head, flexor hypertonicity is present. At four to five days in puppies, the flexor hypertonicity is replaced by extension until three to four weeks of age when the puppies will begin to struggle to escape when held in suspension.
4. The nociceptive threshold is much lower than in adults. This may be due to a lack of some of the descending inhibitory mechanisms found in older animals. The coordination of motor responses to noxious stimuli is not well-developed and the animal may have much wider receptive fields to noxious events. Neurotransmitters may not have reached full function.[8]

Clinical implications. Although the nervous system of the neonate is immature there is no doubt that nociceptive pathways are present and that pain is perceived by the neonate subjected to noxious stimuli. Drugs which might be effective in adults may not be as effective in neonates. Procedures carried out on neonates with insufficient pain control produce greater stress responses than those where analgesia has been provided. A local anesthetic (lidocaine, dose extrapolated from humans) can be used and is very effective; The dose requirement is lower because of the immaturity of the nerves but the neonate does not appear to be at any greater risk of toxic

side effects with a single dose of lidocaine. Bupivicaine is not advised in the neonate due to the risk of cardiotoxicity with overdosage.

The pharmacokinetics of the opioid analgesics are different in the neonate versus the adult. Lower doses of these drugs are required for analgesia at one day of age compared with 34 days (three to four-fold differences).

Metabolism

1. The normal birth weight of the puppy is breed dependent; generally, 500 gm ± 150 gm for a medium breed dog. Birth weights lower than 300 gm in the medium size dog are associated with an increased risk of neonatal mortality. Increased mortality in low birth weight puppies is most likely associated with negative effects of chilling (higher body surface area: mass) and the ability to nurse and maintain glucose concentrations. Generally, there is a similar pattern of growth amongst different breeds of dogs; the most rapid weight gain occurring during the first 12 weeks. Puppies should gain on average 10% of their body weight each day for the first few weeks of life.
2. Unlike their homeothermic adult counterparts, neonates are poikilothermic. However, they have well-developed behavioral heat-seeking responses which enable them to maintain a stable rectal temperature providing sources of heat are available. Shivering and vasoconstrictive reflexes are not functional in the newborn. Physiological responses noted during hypothermia include bradycardia, cardiovascular failure, neuronal injury, and ileus. Normal rectal temperatures in the puppy are 95 – 99 °F (week one), 97 – 100 °F (weeks two and three) and by weaning rectal temperatures approach that of the adult.
3. At birth the neonate must transition from placental support to endogenous food stores for glucose production. During the first three to 24 hours after birth, hepatic glycogen stores decline by more than 50% and there is a shift from glycogenolysis to a mixture of glycogenolysis and gluconeogenesis. For maintenance of blood glucose concentrations, regular feeding is required. In addition to regular nursing, the dam's nutritional state must be adequate to provide for the needs of her puppies.[5]

Clinical implications. The neonate is susceptible to a wide variety of toxic, environmental, infectious and congenital insults; however, the ability for them to respond is limited. One of the first signs of illness in both the kitten and puppy is a failure to gain weight. This finding is often noted well before any other clinical signs of disease are present. Twice daily weighing of neonates during the first week(s) of life dramatically facilitates early detection of illness, ensures adequate intervention in a timely manner to prevent poor weight gain and positively impacts neonatal survival.[1]

Neonatal/pediatric conundrums

Fading Puppies

A fading puppy commonly dies following the onset of rapidly progressive, vague signs of illness. Premortem diagnosis is challenging. Immediate necropsy of a neonate dying without obvious cause is warranted to provide proper veterinary care of the littermates. Clients should be advised to refrigerate (not freeze) deceased neonates and present them promptly for evaluation.

Neonatal bacterial peritonitis with septicemia can cause rapid deterioration of the puppy resulting in death if not recognized and treated promptly. Factors shown to predispose a puppy to septicemia include endometritis in the bitch, a prolonged (often not recognized or reported) delivery/dystocia, feeding of replacement formulas, the use of ampicillin, stress, low birth weight (< 350 gms), and chilling with body temperature <35.5 °C. The umbilicus of neonates should be treated with tincture of iodine immediately after birth to reduce contamination and prevent ascent of environmental bacteria into the peritoneal cavity (omphalitis-peritonitis).

The bacterial organisms most frequently associated with septicemia are *E. coli*, *Streptococci*, *Staphylococci*, and *Klebsiella* spp. Commonly, a decrease in weight gain, failure to suckle, hematuria, persistent diarrhea, unusual vocalization, abdominal distention and pain, and sloughing of the extremities indicate septicemia may be present.

Prompt therapy with broad spectrum, bactericidal antibiotics, optimal nutrition via supported nursing, tube feeding or bottle-feeding, maintenance of body temperature, and appropriate fluid replacement are indicated. The third generation antibiotic, ceftiofur sodium, is an appropriate choice for neonatal septicemia as it alters normal intestinal flora minimally and is usually effective against the causative organisms. The prognosis for septicemic neonates is poor. Failure to respond to antibiotic therapy should prompt consideration of canine herpes virus infection.

Canine herpesvirus (CHV) is a widely recognized and commonly blamed cause of fading puppy syndrome. Premortem and postmortem diagnosis of CHV infection in neonates can be challenging. Typical necropsy findings include multifocal petechial renal hemorrhages. Confoundingly, these can be also be present with bacterial septicemia. Intranuclear inclusion bodies can be difficult to find. Diagnosis by virus isolation or CHV-specific PCR is confirmatory. Treatment has been reported to be unrewarding and recovery is rare. Recovery has been reported to

result in residual cardiac and neurologic damage. Treatment with immune serum from affected dams is reported to be ineffective in infected puppies. One case report of successful treatment with the antiviral drug, acyclovir exists. Successful vaccine development has been hampered by the poor immunogenicity of other herpesviral vaccines developed for other species, as with feline and bovine rhinotracheitis. Neonates of a naïve bitch exposed to CHV during the last two to three weeks of gestation or the first three weeks postpartum are at risk.[9,10]

Acyclovir is an antiviral agent with activity against a variety of viruses including herpes simplex. Acylcovir is preferentially taken up by susceptible viruses and converted into the active triphosphate form, which inhibits viral DNA replication. Acyclovir is poorly absorbed after oral administration and is primarily metabolized by the liver. Acyclovir can increase the toxicity of nephrotoxic drugs. The half-life in humans is approximately three hours. Its use in veterinary medicine is not well established and it should be used with caution and only in situations where indicated. The safety and effectiveness in humans less than two weeks of age is not established. The dose is extrapolated from that for humans.[10]

Juvenile cellulitis

Juvenile cellulitis (puppy strangles) is a progressive, granulomatous, pustular disorder of puppies, most commonly occurring in dogs younger than four months of age, but it is occasionally reported in dogs up to four years of age. The eyelids, pinnae, lips, chin, muzzle, paws, abdomen, thorax, vulva, prepuce and anus can be affected with lesions that fistulate, drain and crust. Lymphadenomegaly, most commonly mandibular and superficial cervical, can be distant from the affected skin sites and is often painful. Pustules and lymph nodes are usually sterile when cultured. Superficial cutaneous flora can be cultured from open, draining lesions. Pyrexia, anorexia, sterile suppurative painful arthritis and an inflammatory hemogram can occur. The diagnosis is confirmed by histopathologic evaluation but is commonly made on the basis of clinical appearance. The predominant inflammatory cell in juvenile cellulitis, characterized by light and electron microscopy and immunohistochemical staining, is an epithelioid macrophage Juvenile cellulitis requires aggressive immunosuppressive therapy early in the course of the disease for resolution and to avoid the sequellae of cicatricial lesions. Traditionally, puppies have been placed on immunosuppressive doses of prednisone (2.2 mg/kg/day), causing concerns with immunization efforts. Griseofulvin therapy offers an apparently effective treatment without the side effects associated with corticosteroid administration, enabling discontinuation of corticosteroids sooner in the course of the disease. It has been reported to be effective as sole immunomodulatory therapy (14.2 to 34 mg/kg PO Q 12 h). Griseofulvin is postulated to induce down regulatory signals within the lesions. The use of griseofulvin as sole therapy could be attempted in early cases. Vaccination of puppies undergoing immunosuppressive therapy is not advised and they must be strictly isolated from sources of infectious disease.[11,12]

Bacterial overgrowth syndrome-associated diarrhea

Pediatric dogs and cats are often presented to the veterinarian for signs referable to the abdominal cavity. Dietary indiscretions, parasitism and infectious disease (primarily viral, less commonly bacterial) account for most of these presentations. Congenital and developmental disorders should also be considered.

Symbiotic colonic bacteria assist digestion. The upper GI tract was once believed to be sterile, but normal colonization of the duodenum, jejunum, and ileum is now appreciated. Bacterial overgrowth syndrome (BOS) occurs when the normally low bacterial colonization in the upper GI tract significantly increases. Neonates are particularly at risk for developing BOS. Mucosal injury resulting from a minor viral or bacterial gastroenteritis can induce BOS in these individuals if a proper post infectious dietary regimen is not followed.

A particular bacterial pathogen has never been implicated; instead, abnormally large numbers of normal or pathological flora appear to cause BOS. Under normal conditions, gram-positive bacteria and fungi colonize the duodenum and jejunum in quantities less than 1×10^5 organisms per milliliter of fluid. Aerobic and anaerobic bacteria colonize the ileum in quantities less than 1×10^8 organisms per milliliter of fluid. This is in sharp contrast to the 1×10^{11} organisms per milliliter of fluid that colonize the colon. Studies of duodenal aspirates have not identified any particular bacteria as a cause of BOS; however, 1×10^5 organisms per milliliter of aspirate fluid is diagnostic for BOS. Usually, abnormally large numbers of anaerobic bacteria and normal florae grow from cultured fluid of patients with BOS.

The following are protective factors that stabilize the number and type of bacteria that colonize the upper GI tract. Abnormalities in these mechanisms put a patient at risk for bacterial overgrowth.

1. Two coordinated motor phenomena produce the continuous propulsive peristaltic action of the upper GI tract. Both the migrating motor complex and the migrating action potential complex clear the upper intestine of unwanted bacteria and undigested substances. Desynchronization of these complexes results in diarrhea and weight loss in animal models. Neonates lack propulsive peristaltic action. Gut motility in neonates results from aboral pressure.

2. Gastric acid normally reduces the proximal small intestine bacteria populations, particularly anaerobic bacteria. The bowel mucosa integrity and mucin layer protect the gut from bacteria. Neonates have reduced gastric acidity.

3. Malabsorption of bile acids, fats, carbohydrates, proteins, and vitamins causes many of the symptoms of diarrhea and weight loss associated with BOS. Anaerobes and *Bacteroides fragilis* actively deconjugate bile acids, thereby preventing proper bile acid function and enterohepatic circulation. Fatty acid absorption is reduced because deconjugated bile acids cannot help micelle formation. Deconjugated bile acids directly inhibit carbohydrate transporters. These unabsorbed sugars ferment into organic acids because of the intestinal flora, which reduces the intraluminal pH and produces osmotic diarrhea. The unconjugated bile acids also damage intestinal enterocytes and induce water secretion by the colonic mucosa.

4. Fat, protein, carbohydrate, and vitamin malabsorption result from poor enterocyte function and bacterial transformation of nutrients into nonabsorbable and toxic metabolites. Toxic metabolites damage the intestinal mucosa. Malabsorption and enterocyte dysfunction further degrade the health of the gut by reducing local and systemic nutrition delivery.

Treatment of BOS is aimed at reducing the damage caused by malabsorption and restoring nutritional health and normal gut flora. Prompt recognition and treatment can prevent the development of severe malnutrition. The antimicrobials of choice for therapy of BOS-associated diarrhea are ampicillin or amoxicillin in the pediatric patient (due to the neurotoxicity associated with metronidazole overdosage).[14]

Anasarca

Anasarca, a lethal congenital edema, can occur with or without concurrent cardiovascular abnormalities. Generalized subcutaneous edema, with intrathoracic and intraperitoneal fluid accumulation is present. Congenital hereditary lymphedema causes edema of the extremities and sometimes head, and is associated with morphologic lymphatic abnormalities. Prepartum ultrasonographic evaluation of the fetuses can be used to screen for this disorder. Dystocia can result due to fetal oversize. Anasarca is a problem common in Bulldogs, but recognized in other breeds as well (Labrador retriever). It is suspected to have a heritable component. Its exact pathophysiology in the dog is not understood. The genetics are not known; anasarca is thought to be inherited as an autosomal dominant trait. There are multiple anecdotal remedies, none proven or reported in the scientific literature. It is debated and discussed on theriogenology list serves and on the layman's internet exhaustively. As well as causing dystocia, anasarca usually results in stillborn puppies or puppies needing to be euthanized. Some veterinarians promote various therapies, usually doomed to failure. Diuretic therapy of affected neonates can sometimes cause slight normalization, but euthanasia is usually indicated if the neonate is not stillborn. Environmental, dietary, and pharmacologic contributory factors are not scientifically defined. Anasarca has been recognized for many years, yet its incidence remains unchanged. An attempt to recognize the presence of anasarca prepartum with ultrasonography should be made in bitches with a history of affected puppies or in breeds with high incidence, due to the higher incidence of dystocia associated with the syndrome.

"Swimmer" puppies

Swimmer puppies fail to develop normal ambulation at ten to 14 days of life, moving instead by paddling their limbs laterally and caudally. Compression and deformation of the sternum and thorax occur concurrently. Obese puppies from small litters, commonly raised on relatively slippery surfaces are predisposed. Treatment should be instituted immediately upon diagnosis, consisting of caloric restriction, physical therapy, and improved traction in the nest box. If diagnosed early (three to five weeks of age) the condition is reversible and does not require binding of puppies.

Puppy vaginitis

Puppy vaginitis is characterized by an apparently healthy female puppy presented with mucoid vulvar discharge that is usually white to yellow, and sometimes copious. The discharge can be accompanied by mild perivulvar dermatitis. The puppy is not typically attentive to the discharge, and there is not any associated change in urinary behavior (dysuria or polakiura). Clients often have a difficult time deciding if a puppy has normal urinary behavior or not. The age of onset ranges from six weeks to puberty, the duration is from days to months, and the disorder is often intermittent.

Cytologic examination of the discharge finds suppurative inflammation. Vaginal cultures (aerobic) generally fail to grow anything but normal flora in small numbers, similar to unaffected littermates. A urinalysis, acquired by cystocentesis, is characteristically normal (a decreased urine specific gravity is typical for young dogs lacking adult concentrating abilities), and the urine culture negative. The clinician needs to perform enough diagnostics to rule out more significant causes of vulvar discharge and feel comfortable with the diagnosis of benign puppy vaginitis.

The specific etiology of puppy vaginitis is unknown. An imbalance of juvenile vaginal glandular epithelium is postulated. The condition is reported in the literature to resolve both with puberty and with ovariohysterectomy, two very different events endocrinologically, therefore neither likely to truly cause resolution. Puppy vaginitis diminishes with maturity. The term "puppy vaginitis" is a misnomer, as it is asymptomatic and not indicative of inflammation. Important rule-outs (some of which are associated with inflammation) include urinary tract infection, urinary incontinence with associated mucosal scalding, the onset of the initial estrous cycle, vaginal foreign bodies (i.e. foxtails) and urogenital anatomic anomalies (ectopia, disorders of sexual differentiation, significant strictures). Cleansing the perivulvar area with a gentle solution (non-alcoholic otic preparations or "baby wipes"), benign neglect and tincture of time are advised.[14,15]

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Neonatal and pediatric ultrasonography- part I

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Introduction

Small animal patients are commonly presented to the veterinarian because of signs referable to the abdominal cavity due to congenital anomalies, dietary indiscretion, parasitic infestation and infectious or inflammatory disease. Abdominal ultrasound provides valuable clinical information about the peritoneal cavity, great vessels, abdominal viscera and lymph nodes obtained in a non invasive fashion, with no confirmed adverse biologic effects, and usually not necessitating sedation or anesthesia. This paper reviews the techniques for performing the pediatric abdominal ultrasound scan.

Abdominal ultrasound provides useful data in a short period of time. The normal paucity of intra-abdominal fat in pediatric patients results in less informative abdominal radiography, but actually improves ultrasonographic imaging. (Abdominal fat attenuates the ultrasound beam.)[1] Image quality is improved with small patient size as a higher frequency scanhead can be employed. Acquisition of special equipment for pediatric ultrasonography is usually not necessary as scanheads selected for small animal (especially feline) clinical use are appropriate for most pediatric cases.

Small animal patients are best evaluated using an ultrasound machine equipped with a curvilinear variable frequency scanhead (6.0-8.0 MHz). Many portable machines now have available a high frequency linear scanhead (8.0 -10.0 MHz) which will improve quality and also allow evaluation of smaller regional anatomy (thyroid, parathyroid, cryptorchid testes).[2]

Keywords: Ultrasound, pediatric, abdominal, neonatal

Preparation

The small animal patient is placed in dorsal recumbency within a padded V-trough, and is gently restrained by assistant(s) holding the forelimbs and hindlimbs. Sedation is rarely required for the basic abdominal scan unless marked pain or apprehension is present. Allowing the patient to become accustomed to this restraint before clipping the hair or initiating the scanning process usually minimizes struggling and the resulting aerophagia.[3]

To prepare the pediatric patient for abdominal ultrasonography, the cranioventral abdominal hair is clipped using a No. 40 blade. Wetting the skin with water, tincture of zephiran or 70% isopropyl alcohol, followed by a liberal application of ultrasound gel, permits the best acoustic coupling of the scanhead to the patient and improves the image obtained. Some pediatric patients have scant ventral haircoats that do not require clipping. Care should be taken to avoid excessive chilling of pediatric patients. Chilling can occur secondary to the application and evaporation of room-temperature wetting agents. Electric warming devices (warm water blankets) may cause electronic interference with the ultrasound equipment; warm water bottles or their equivalent are superior.[1]

Patient preparation includes fasting as much as is safely possible given the animal's age and size. Fasting minimizes obscurement of the liver imaging by gastric ingesta and of other abdominal viscera by gastrointestinal gas accumulation. Preventing urination immediately prior to the examination allows urine to accumulate within the urinary bladder lumen and permits better evaluation of this structure. [4]

Serial evaluations can provide useful information when the clinical status of the small animal patient has changed. Indications for serial ultrasonographic evaluation include clinicopathologic deterioration, progressive lethargy or obtundation, acute pain, changes in abdominal palpation findings and refractory vomiting or diarrhea. All warrant repeat evaluation for signs indicating the development of intussusception, perforation and/or peritonitis.

The normal abdomen

Regardless of the clinical history, the abdomen should be evaluated methodically with the animal in dorsal decumbency. Realize the sector image you are looking at on the monitor screen is perpendicular to that ultrasound beam. You are viewing the sagittal image from the side of the animal and the transverse image from the rear of the animal. (fig 1a-c, fig 2a-c).[1]

Place the scanhead under the xyphoid with the beam in sagittal plane. Visualization of the liver is achieved by fanning the beam from right to left. The gall bladder is seen on the right; the left liver lobes are seen ventral and sometimes caudal to the stomach. Turning the beam to transverse allows for visualization of the liver between stomach and gall bladder. This view is useful for evaluation of the hepatic border, echogenicity of hepatic parenchyma and portal architecture. The portal vessels have very echogenic walls.[5] (fig 3)

Resuming the sagittal plane, scan to the left of the dog past the stomach to the spleen. The spleen will be visualized ventrally in the near field. Splenic border, parenchyma and shape should be evaluated.[6] (fig 4) Following the spleen transversely down the left body wall, you will image the left kidney. (fig 5)

Once visualization of the kidney is achieved, turn the transducer to the sagittal plane and evaluate the renal border, cortical echogenicity and pelvic architecture. Dilatation of the renal pelvis is best seen in the transverse plane. Normal ureters are not usually visible ultrasonographically.[1,7] The left adrenal gland is located medial to the cranial pole of the kidney. In a sagittal plane, maintaining strong hand pressure, scan medially to visualize the linear aorta and the renal artery. The left adrenal is located cranial to the left renal artery and caudal to the left cranial mesenteric artery. The left adrenal gland is imaged as a bi-lobed structure with the phrenicoabdominal vein at its waist.[1] (fig 6)

With the transducer in a transverse plane in the midabdomen, scan caudally to image a large hypoechoic structure, the urinary bladder. Evaluate bladder wall and lumen contents, and, dorsal to the bladder, the major vessels (caudal vena cava and aorta). Sublumbar lymph nodes will be seen at the aortic bifurcation into the iliac arteries, adjacent to the bladder wall. (fig 7) Sagittal scanning of the urinary bladder caudally will allow visualization of the urethra (and prostate in the male).[4] (fig 8)

Continuing in methodic fashion, the right kidney will be imaged at the edge of the right ribcage adjacent to the renal fossa of the liver. The right kidney should be evaluated as was the left with respect to renal border, cortical echogenicity and pelvic architecture. By scanning sagittally between the right kidney and the caudal vena cava with a fanning technique, the right adrenal gland is visualized just lateral to the caudal vena cava. In a transverse plane, the duodenum is imaged lateral to the right kidney.[1] (fig 9)

At the cranial end of the right kidney medial to the duodenum will be the right limb of the pancreas. The right pancreatic limb is identified by visualizing the caudal pancreaticoduodenal vein within the structure. (fig 10) Turning to the sagittal plane, follow the pancreas, scanning medially to the angle of the body and left limb, or sagittally scan the caudal border of the stomach. The pancreatic body is seen caudal to the stomach, cranial to the splenic vein. The left pancreatic limb is found caudal to the splenic vein and midline to the cranial pole of the left kidney.[5] (fig 11)

Returning to the transverse plane in midabdomen at the mesenteric root, scan for mesenteric lymph nodes and small bowel wall changes. Scanning in a uniform serpentine fashion, 2-3 passes may be required to evaluate the entire bowel. Normally, the small bowel appears sonographically as four distinct layers. (fig 12, 13) The bowel lumen is hyperechoic, as gas and ingesta are compressed. The layer just outside the lumen is the mucosa; it is hypoechoic and normally the thickest appearing section. Outside the mucosa is the submucosa, it is hyperechoic to the mucosa and about one third the thickness. The muscularis, the bowel muscle layer, is outside of the submucosa and appears as a very thin hypoechoic black line. The outermost serosal layer is hyperechoic.[1] (fig 14)

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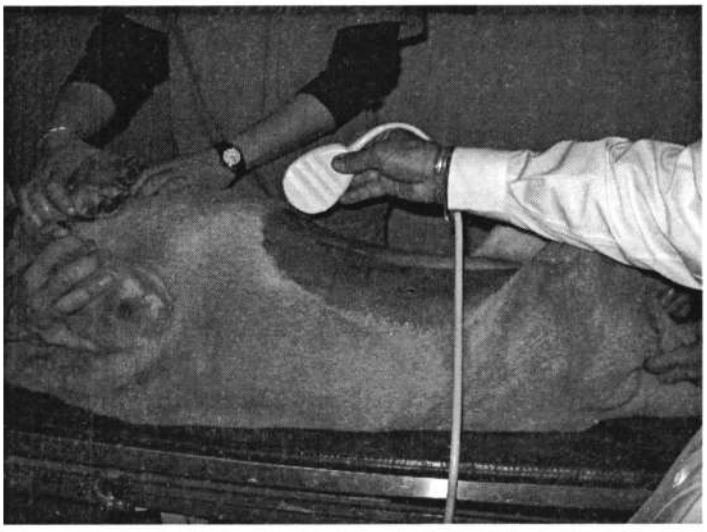

Fig 1a The correct sagittal scanhead placement.

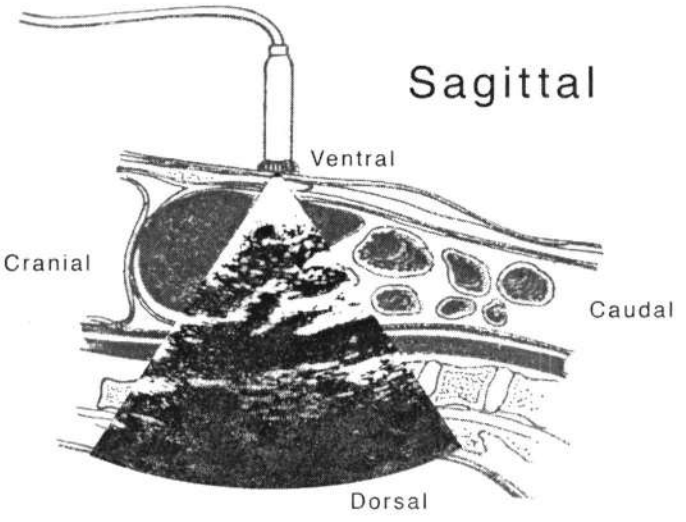

Fig 1b Schematic of the resultant ultrasound beam in the abdomen.

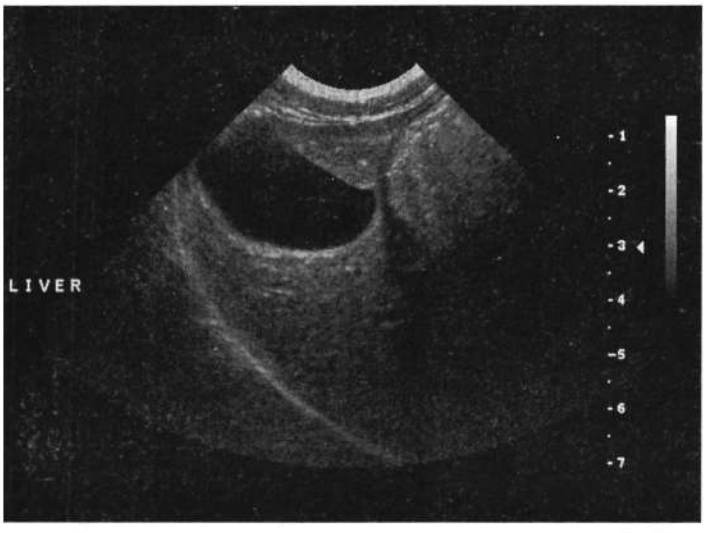

Fig 1c The resultant sagittal image of the liver and gall bladder.

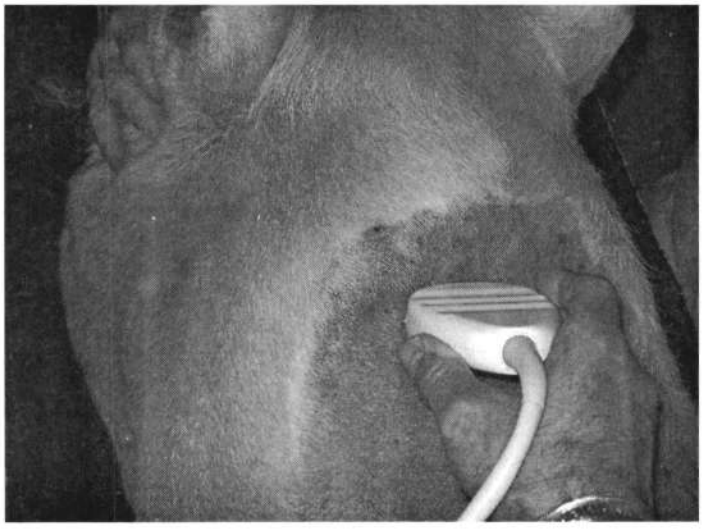

Fig 2a The correct transverse scanhead placement.

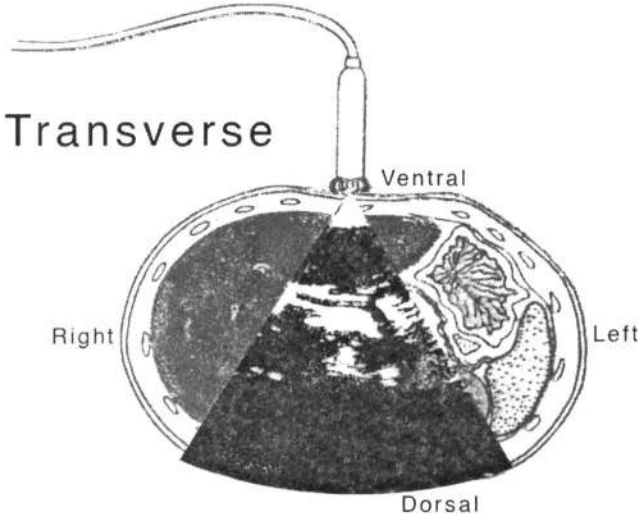

Fig 2b Schematic of the resultant ultrasound beam in the abdomen.

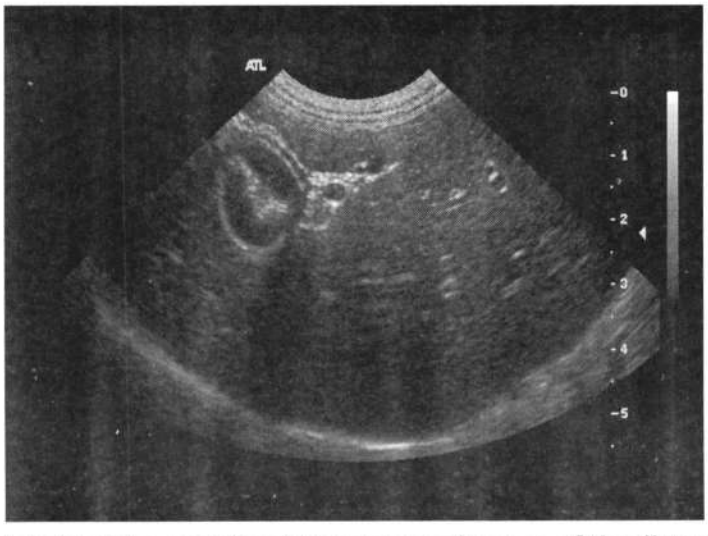

Fig 2c The resultant transverse image of the liver and duodenum.

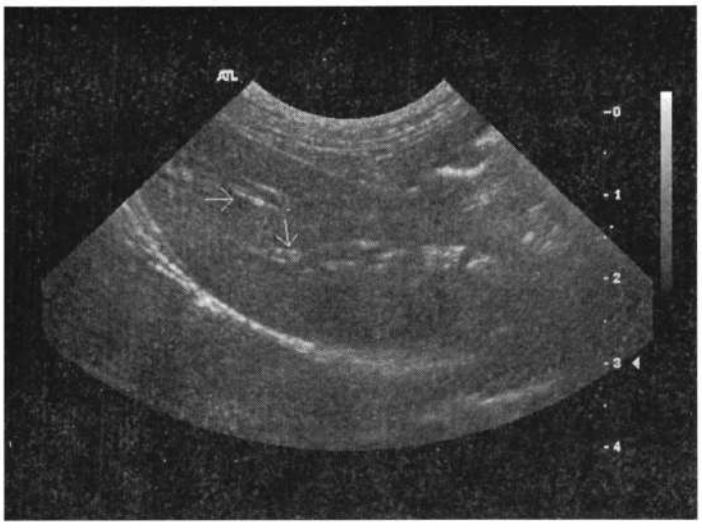

Fig 3 Normal hepatic parenchyma with white walled portal vessels (arrows).

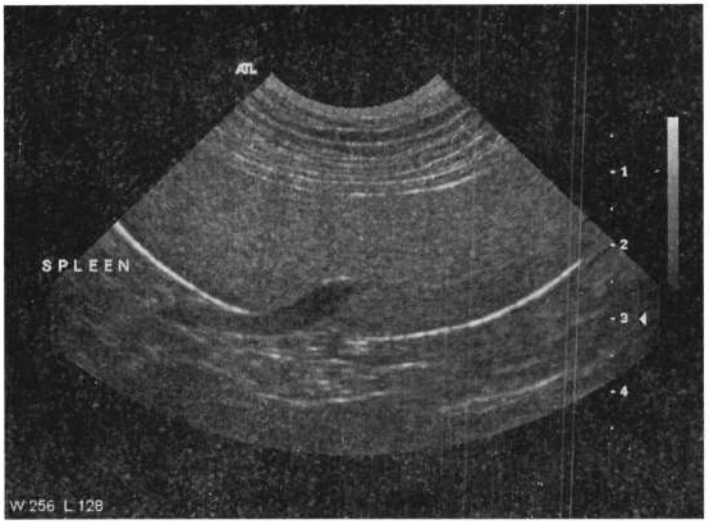

Fig 4 Normal transverse spleen; splenic vein exiting through the splenic capsule.

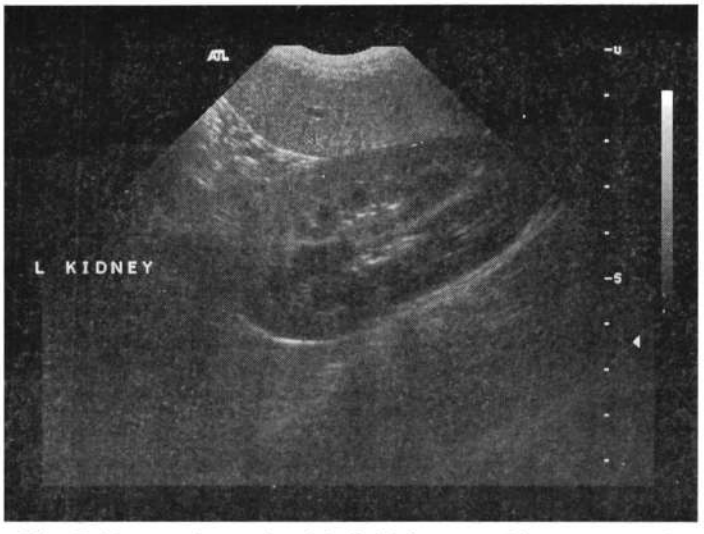

Fig 5 Normal sagittal left kidney, with a normal cortico-medullary interface.

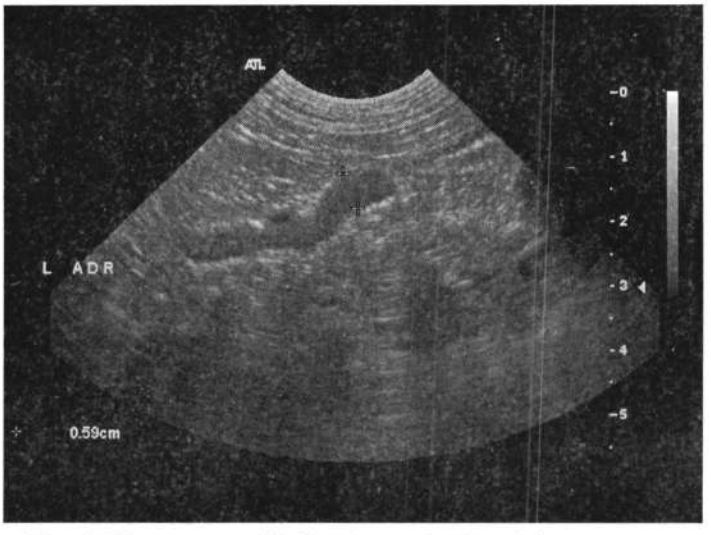

Fig 6 The normal left adrenal gland (cursors at caudal pole) showing the phrenicoabdominal vein at its waist.

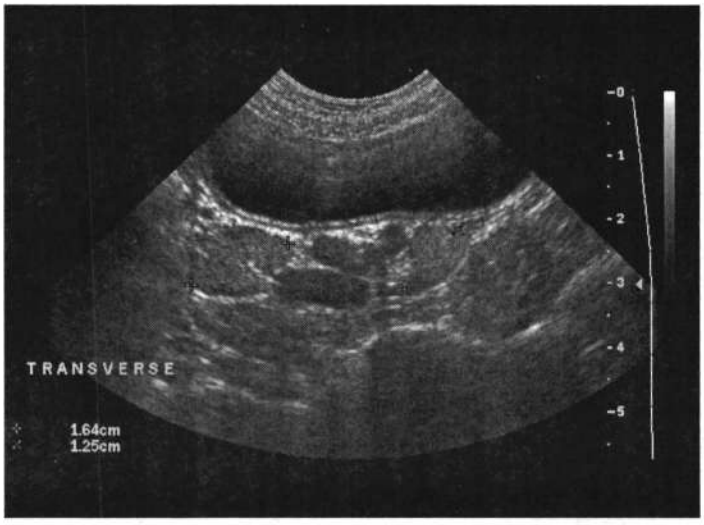

Fig 7 Transverse view of the aortic bifurcation into the iliacs, showing the location of the sub-lumbar lymph nodes (cursors).

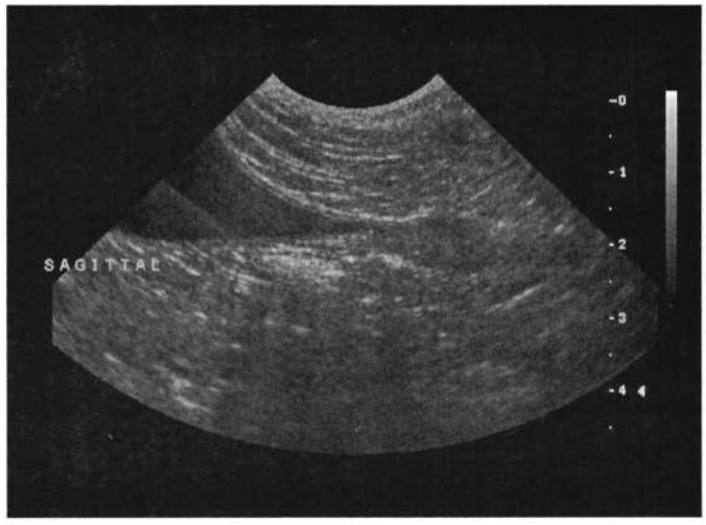

Fig 8 Transverse image of normal neutered male canine prostate.

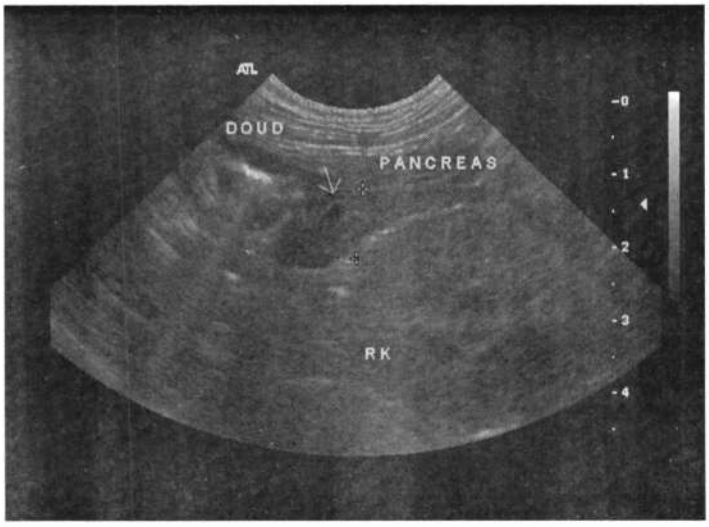

Fig 9 Transverse image of the duodenum lateral to the right kidney. The normal right pancreatic limb (cursors) is visible between these two structures. The caudal pancreatoduodenal vein is seen (arrow).

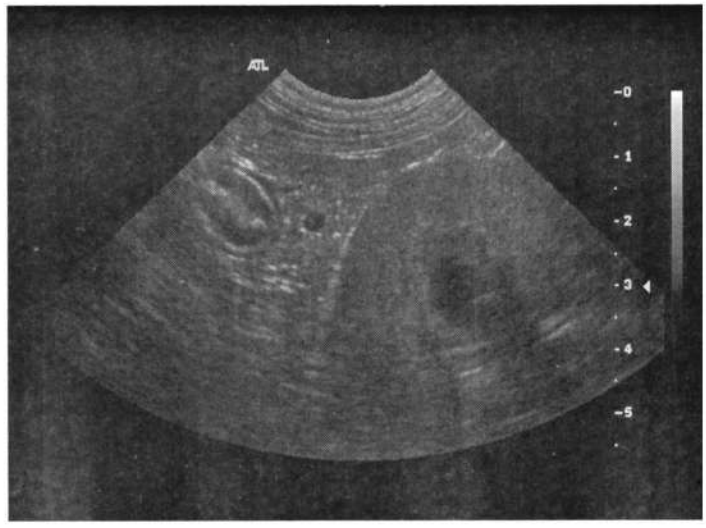

Fig 10 The caudal pancreatoduodenal vein is seen as a hypoechoic oval structure within the right limb of the pancreas.

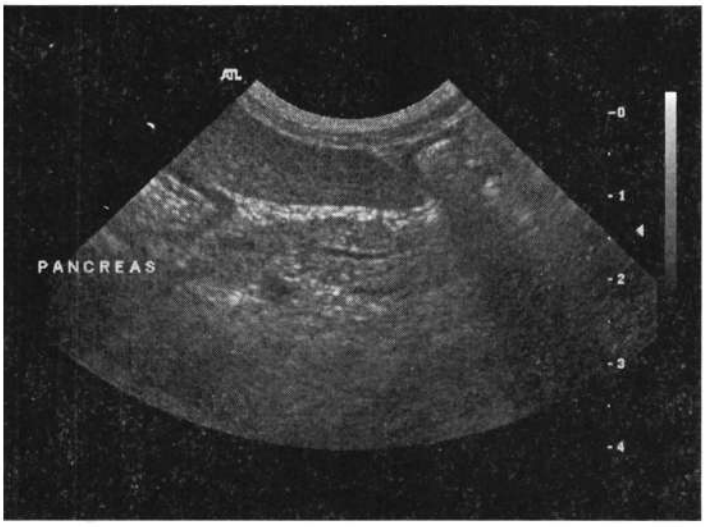

Fig 11 The pancreatic body and left limb are caudal to the gastric wall, dorsal to the spleen, and medial to the left kidney. Note the splenic vein positioned dorsal to the pancreas.

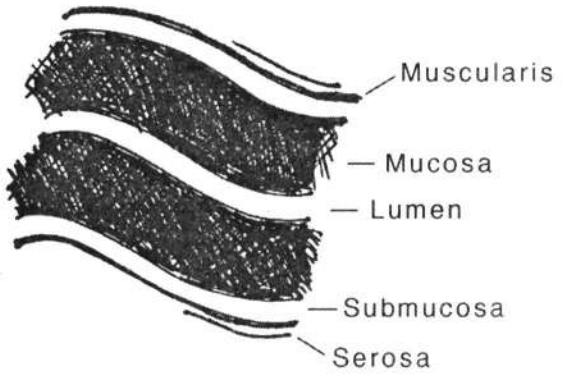

Fig 12 Schematic representation of the normal small bowel wall layers.

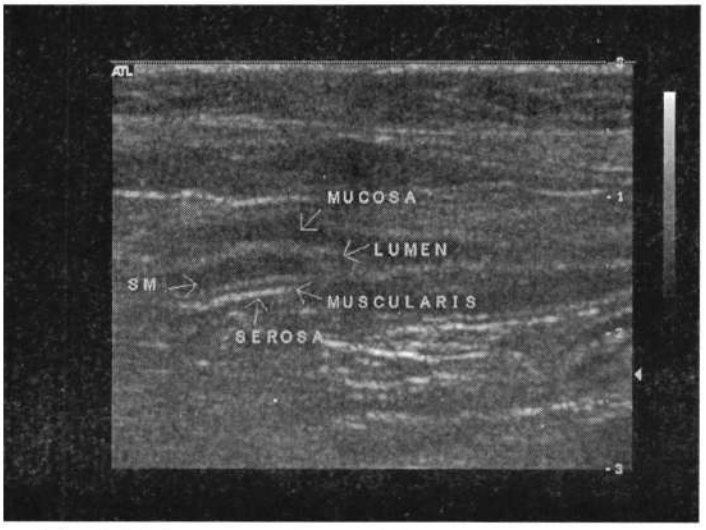

Fig.13 The normal small bowel wall layers.

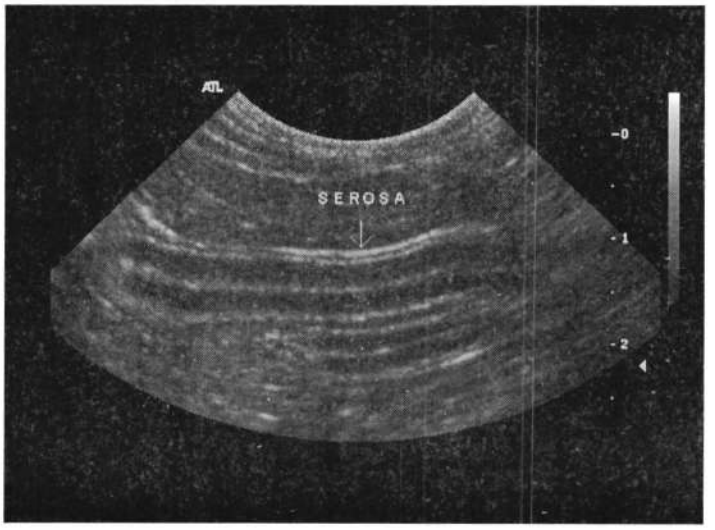

Fig 14 Image of normal small bowel serosa.

Neonatal and pediatric ultrasonography-part II

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Introduction

Pediatric patients are commonly presented to the veterinarian because of signs referable to the abdominal cavity. Presenting signs can be due to congenital anomalies, dietary indiscretion, parasitic infestation and infectious disease. Abdominal ultrasound examination is a particularly useful diagnostic tool in the pediatric patient because it is noninvasive and can usually be performed without sedation or anesthesia. Ultrasonography provides valuable clinical information about the peritoneal cavity, great vessels, abdominal viscera and lymph nodes and thus greatly facilitates diagnostic differentiation between congenital and acquired disorders.[1] Ultrasonographic techniques will be described in this paper.

Keywords: Ultrasound, neonatal, congenital, acquired, pediatric

Disorders of urogenital development

In the past, veterinary pediatric ultrasonography has been hampered by the small size of neonatal organs. Recent advances in pediatric veterinary ultrasonography are encouraging. Abdominal ultrasound can facilitate the diagnosis of congenital urogenital disorders because ectopic, distended ureters and changes in renal architecture are usually readily imaged.[2] The presence and location of cryptorchid testes can often be detected with ultrasound.[1] Ultrasonographic examination of the bladder disclosing urolithiasis can provide information suggesting congenital hepatic vascular anomalies. Ammonium biurate urolithiasis is suggestive of hyperammonemia, common with portosystemic shunt disorders (described below).[3,4]

The most common familial disorders in cats and dogs include renal agenesis, renal dysplasia, polycystic kidneys, renal amyloidosis, basement membrane disorders, and tubular dysfunction (Fanconi's syndrome).[5]

Renal agenesis

Congenital renal agenesis resulting in the absence of a kidney can be confirmed with ultrasound. The contralateral kidney typically has normal internal anatomy, but is enlarged as a consequence of obligatory hypertrophy. Renal function of the pediatric patient does not equate that of the adult until 4-6 months of age, therefore compensatory renomegaly may not be apparent until that time.[3]

Renal dysplasia

Until reliable genetic markers are marketed and thus available for the various breed specific congenital renal dysplasias(i.e. Persian cats) , ultrasound provides the best method of screening young dogs and cats for these likely heritable disorders. Early ultrasonographic screening is possible in platycephalic breeds in which morphologic changes are grossly evident (i.e., Cairn Terriers, German Shepherd Dogs).[3,5] (fig1)

Ectopic ureter

Congenital ectopic placement of a distal ureter into the urethra, vestibule or vagina is usually associated with ureteral dilation with or without renal pelvic dilation. Dilation of the ureter improves the sensitivity of the ultrasound study; however, the diagnosis can be elusive. Visualization of a nonvascular fluid filled structure with a hyperechoic wall passing dorsal to the urinary bladder, or obvious insertion of the structure into the proximal urethra suggest the diagnosis. Visualization of the ureteral jets in the bladder suggests normalcy, however some ectopic ureters insert initially into the bladder and additionally tunnel distally to terminate in an abnormal site. Visualization of the dilated ureter usually occurs near the urinary bladder. (fig 2) Visualization of the bladder neck and proximal urethra may be obscured by pubic bone, making identification of such termination difficult.[1,2]

Hydronephrosis can eventually result from an uncorrected ectopic ureter due to flow impedance at the abnormal site of insertion. (fig 3) Urinary tract infection is commonly associated with ectopia, due to accompanying urethral sphincter mechanism anomalies. If not detected and treated, urinary tract infection can progress to pyelonephritis and ureteritis. Infection and its associated inflammation in the tract can further alter the ultrasonographic appearance of the kidneys, bladder, ureters and urethra.[1]

Contrast enhanced computed tomography is the most sensitive and specific modality for the diagnosis of ectopia, but, like double contrast radiography, requires anesthesia, making initial evaluation with ultrasound desirable when ectopia is suspected clinically. The condition is thought to be heritable (mode not known), and is more commonly symptomatic in females due to the greater strength of the male urethral sphincter and longer length of the urethra.[5]

Ureterocele

A ureterocele is an uncommon congenital dilation of the ureter near the bladder, appearing as a cystic structure within the bladder lumen or wall. (fig 4) The ureterocele occurs most commonly in association with an

ectopic ureter. Diagnosis can be made by scanning the urinary bladder in the transverse plane and watching for strong peristalsis of the adjacent ureter.[1]

Patent urachus

The urachus permits the flow of urine from the bladder into the allantoic sac of the fetus, and normally atrophies at birth. A patent urachus in the neonate is characterized clinically by urine dribbling from the umbilicus. The fluid filled urachus can be identified ultrasonographically, extending cranially from the cranioventral bladder wall. If an incompletely patent urachus is present in the neonate, a urachal diverticulum may result, seen as a divot in the apex of the bladder. (fig 5) Urachal diverticula can predispose the bladder to recurrent infection because of abnormal bladder flow in the region, surgical excision can be indicated.[1]

Cryptorchidism

Ultrasound identification of cryptorchid testis(es) can confirm cryptorchidism in pediatric patients with bilateral involvement whose neutering status is unknown. Ultrasonographic localization of undescended testes can assist the surgeon in planning the approach (i.e., inguinal vs. cranial abdominal). A retained testis can be positioned anywhere between the ipsilateral kidney and the scrotum. A systematic evaluation of the region from the caudal renal pole to the inguinal canal can identify an oval, homogenously echogenic structure with a mildly hyperechoic border representing the parietal and visceral tunics. The epididymis is usually distinctly less echoic than the testicular parenchyma, as in the scrotal testis. The cryptorchid testis will maintain the anatomic structure, the median testes (a hyperechoic slash), and normal testicular echogenicity despite being reduced in size as compared to a scrotal testis.[1] (fig 6)

Ultrasonography is also the procedure of choice to detect undescended testicles in pediatric or adult dogs and cats. Clinical evaluation via serum LH concentration or testosterone stimulation tests can increase support the diagnosis. An ultrasound examination may also detect nonpalpable scrotal testicular tumors and neoplastic transformation in abdominal testes.[1] (fig 7)

Disorders of digestive system development

Hernia

Congenital peritoneopericardial diaphragmatic hernias occur in both the dog and cat; ultrasonography provides an additional modality for their diagnosis. As with other diaphragmatic hernias, careful evaluation for continuity of the echogenic diaphragm differentiates a true hernia from mirror image artifacts. Evaluation of the pericardial contents can be made from the subcostal (across the liver) or intercostal (using the heart as an acoustic window) approach. Abnormal pericardial contents can include falciform fat, liver, gall bladder and/or intestines. Congenital inguinal and scrotal hernias can similarly be confirmed by ultrasonographic identification of intestines in the subcutaneous space of the affected groin. This can be a dynamic finding. Mesenteric fat may alternatively be entrapped through the hernia.[1,6] (fig 8)

Congenital hiatal hernias are more difficult to confirm with ultrasound because of the inherent difficulty imaging the gas filled stomach and the intermittent nature of the disorder. Stomach wall with characteristic rugal folds can be imaged crossing the diaphragm into the thoracic cavity. Fluoroscopic evaluation can be more informative in these cases.

A developmental anomaly resulting in extrusion of a portion of the gastrointestinal tract outside of the body wall, occurring within the umbilical canal (omphalocele) or lateral to the umbilical canal (gastroschisis), has been reported in humans and occurs in both dogs and cats. The condition is usually hopeless in small pediatric patients presented to the veterinarian hours after birth; however, a 30-70% survival rate is reported in humans with immediate post partum surgical intervention. Diagnosis is made pre partum with abdominal ultrasound, based on the recognition of fetal gastric wall (rugal) structures or intestinal contents in an abnormal location. Earlier surgical intervention before inevitable septic contamination occurs may improve the prognosis in veterinary patients.[1,5]

Enteric anomalies

Pyloric stenosis secondary to hypertrophic gastritis has been reported in a pediatric dog. Focal circumferential thickening of the pylorus primarily involving the muscularis is typical.

Enteric duplication or agenesis can be confirmed ultrasonographically in pediatric patients. Duplication is rare, can occur anywhere in the intestinal tract and the clinical signs may be nonspecific. A fluid filled juxtaintestinal formation with variable peristalsis and contents can be seen. Enteric agenesis usually results in severe clinical signs in the neonatal period. Ultrasonographic findings usually include marked fluid and gas distention of bowel proximal to the defect.[5]

Several breeds of dogs have a reported genetic predilection to small intestinal disease. Normally, the small bowel appears sonographically as four distinct layers. The bowel lumen is hyperechoic, as gas and ingesta are compressed. The layer just outside the lumen is the mucosa; it is hypoechoic and normally the thickest appearing section. Outside the mucosa is the submucosa, it is hyperechoic to the mucosa and about one third the thickness. The

muscularis, the bowel muscle layer, is outside of the submucosa and appears as a very thin hypoechoic black line. (fig 9, 10)

An immunoproliferative enteropathy is seen in the Basenji breed which is characterized by lymphangectasia, intermittent diarrhea, weight loss, hypoalbuminemia and hyperglobulinemia, and lymphoplasmacytic mucosal infiltrates throughout the GI tract. Histopathology is diagnostic, however abdominal ultrasonography can identify bowel in which disruption of the normal layering has occurred. Chinese Sharpei dogs have been identified with a lymphoplasmacytic-eosinophilic infiltrative enteropathy that is characterized by poor weight gain, weight loss, or intermittent diarrhea episodes, with onset of signs typically between 2 to 6 months of age. Infiltrative enteropathies can be characterized ultrasonographically as having changes in the normal bowel wall layering.[5]

Portosystemic shunt

Portosystemic shunts (PSS) are congenital malformations of the hepatic portal venous drainage system and can have either a familial, i.e. genetic, or random occurrence. Congenital PSS can be either intrahepatic or extrahepatic; breed predilections for extrahepatic shunts include Yorkshire terrier, Maltese, Poodle, Miniature Schnauzer, Dachshund, Lhasa Apso, Pekingese, Pug, and Shih Tzu, whereas intrahepatic shunts are more commonly identified in large breed dogs such as Golden Retrievers, German Shepherds, Irish Wolfhounds, Irish Setters, and Samoyeds. PSS are uncommon in cats.

Ultrasonography provides a rapid and noninvasive method for screening patients suspected to have congenital PSS. Although scintigraphy (transcolonic portal scintigraphy or transplenic portography) is considered the most reliable noninvasive method of documenting a PSS, its availability is limited to specialty and university practices, and its use dictates special handling of the radioactive patient for at least 12 hours. Mesenteric portography, although more invasive and requiring general anesthesia, is a highly reliable method of confirming and localizing PSS.[1,7]

Abdominal ultrasonography is a useful diagnostic tool and is routinely done when a PSS is suspected. (fig 11,12) It is non-invasive and requires no anesthesia however diagnostic accuracy is highly operator dependent, and the PSS will be confirmed in only approximately 60-80% of cases. The liver may be small and difficult to image in patients with congenital portosystemic shunts. Imaging the liver from the standard ventral approach can be improved in some cases by using the left ventral intercostal and right dorsal intercostal approaches. The presence of ascites can facilitate the study, as can adding fluid to the stomach, and positioning the patient to shift gas away from the scanhead and shift abdominal organs caudally.(fig 13) Ultrasound evaluation of portosystemic anomalies can be facilitated by positive pressure ventilation under anesthesia for the same reason. Cystic calculi, most commonly ammonium biurate, should increase the clinical suspicion of PSS. Urinary bladder calculi (radiolucent and radiopaque) produce a strong acoustic shadow when viewed ultrasongraphically.[1] (fig 14)

Post operatively, ultrasound can be used to evaluate portal blood flow following surgical banding or coil embolization. Extrahepatic shunts most commonly arise from the portal vein, splenic vein or left gastric vein in the dog, and from the left gastric vein in the cat. (fig 15) Identification of a shunting vessel emptying into the caudal vena cava is difficult but confirmatory. Intrahepatic shunts can be more difficult to identify because of patient size, bowel gas and liver size. Clipping the hair coat intercostally on the right can allow for transverse vessel stacking (of the aorta, vena cava and portal vein) and allow visualization of ductal shunts. There can be right and left shunting of the ductus.[1,7]

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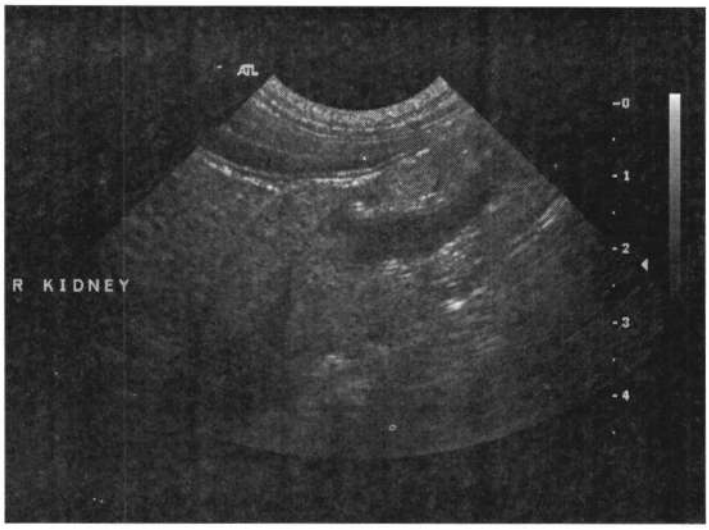


Fig 1 Renal dysplasia, 9 month old dog. The left kidney is misshapen, with cortical thickening and pelvic dilation.

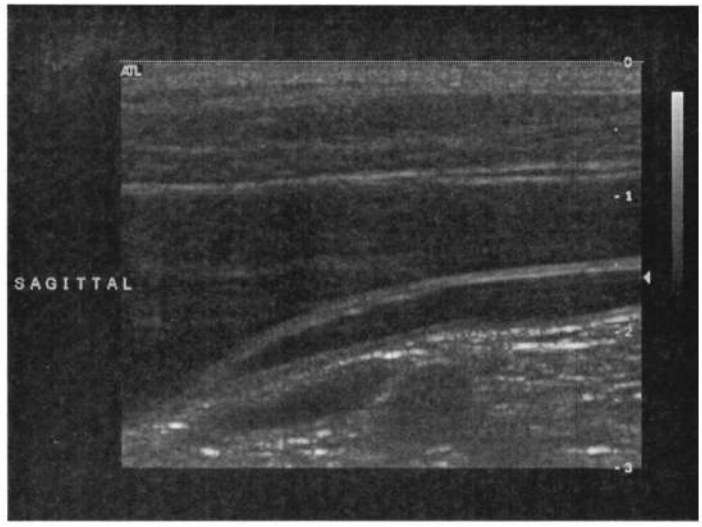


Fig 2 Ectopic ureter. A sagittal image of the urinary bladder shows an ectopic ureter positioned just dorsally. The ureter has a thicker, more hyperechoic wall than the major blood vessels in this region, and can show propulsion during real time evaluation.

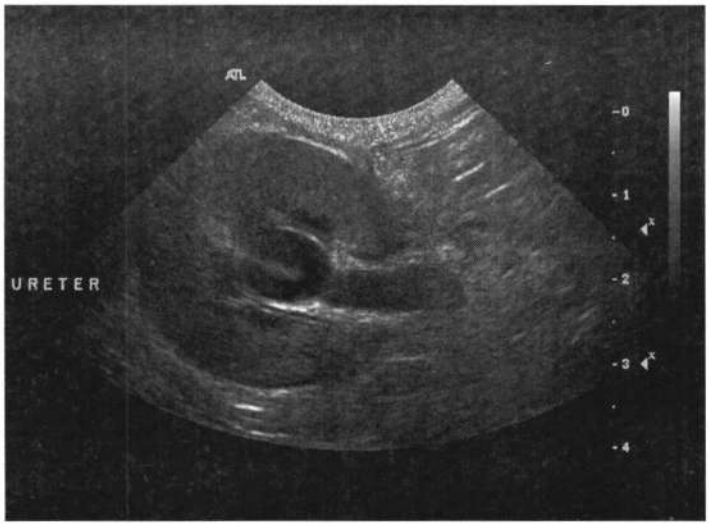


Fig 3 Hydronephrosis, proximal hydroureter, transverse image.

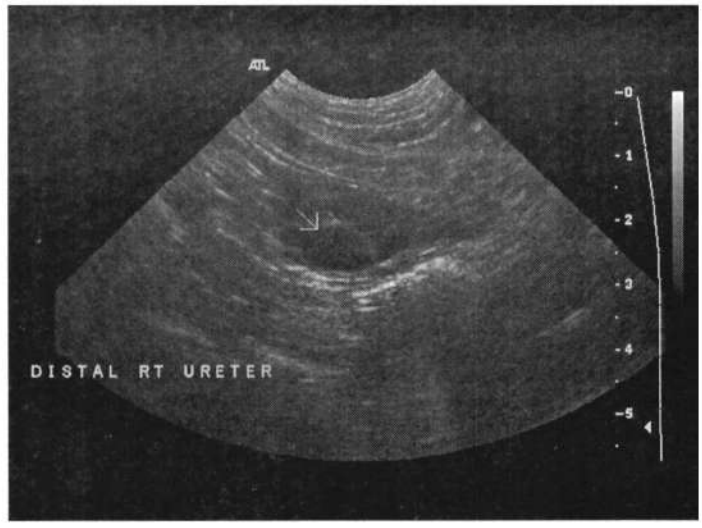


Fig 4 Ureterocele. A transverse image of the urinary bladder shows the intramural extension of an ectopic ureter, the Ureterocele (arrow). Propulsion can be seen during real time evaluation.

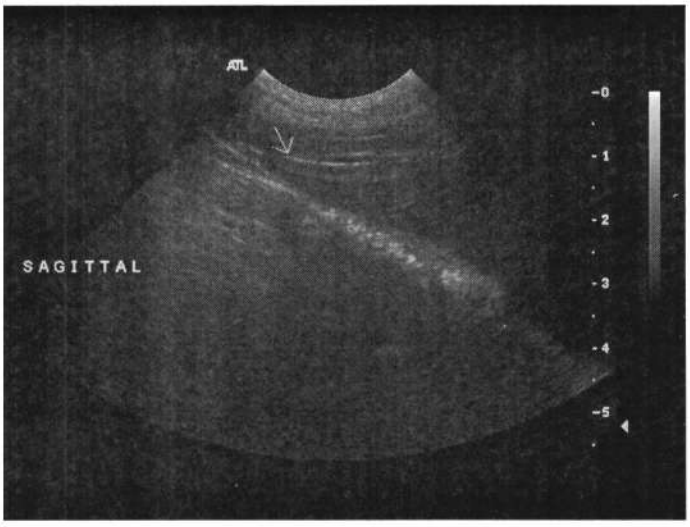


Fig 5 Sagittal view of the urinary bladder; the arrow indicates the site of a urachal remnant.

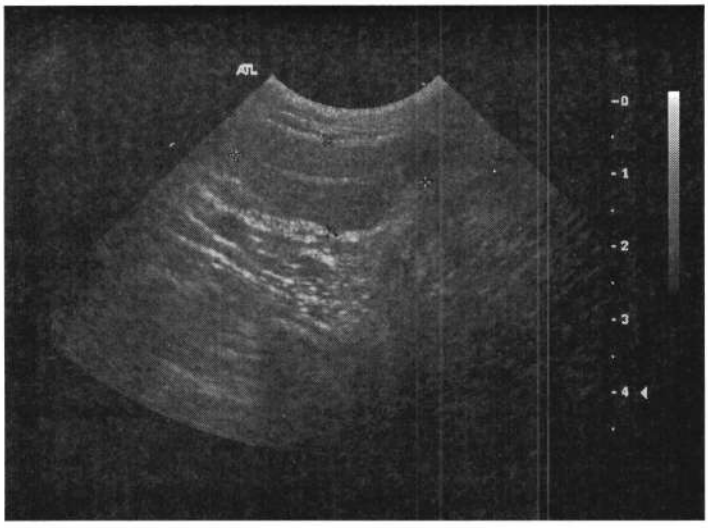


Fig 6 Cryptorchidism. A sagittal image of an intra abdominal testicle (cursors); the testicle is identified by the presence of the mediastinum testis.

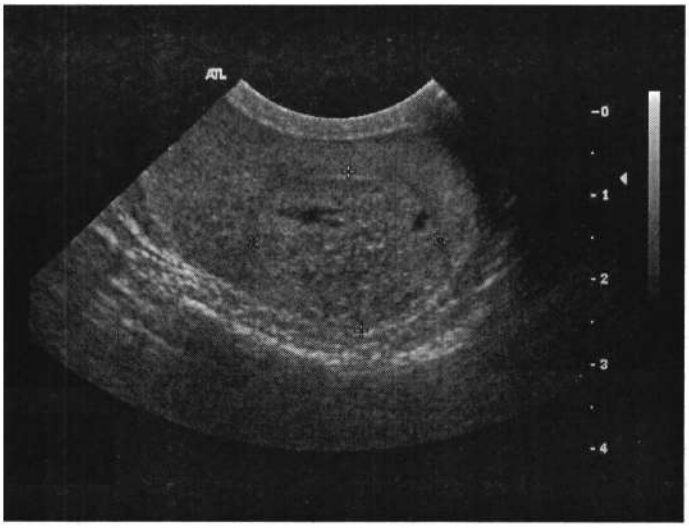


Fig 7 Seminoma. A hyperechoic mass (cursors) with mixed echogenicity is present within the testicular parenchyma.

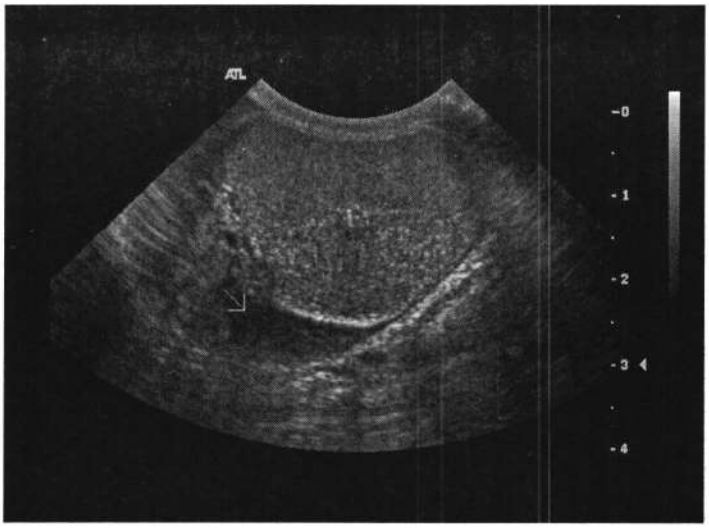


Fig 8 Scrotal hernia, omental tissue and fluid are present within the scrotum.

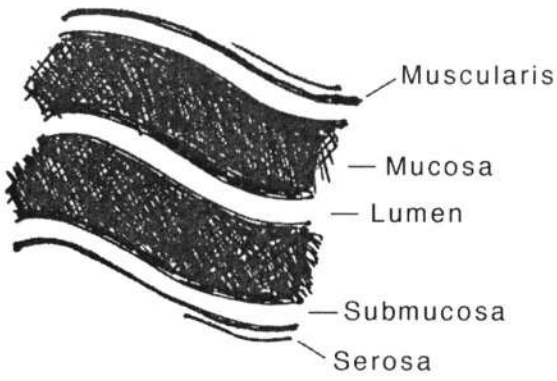


Fig 9 Schematic representation of the normal small bowel wall layers.

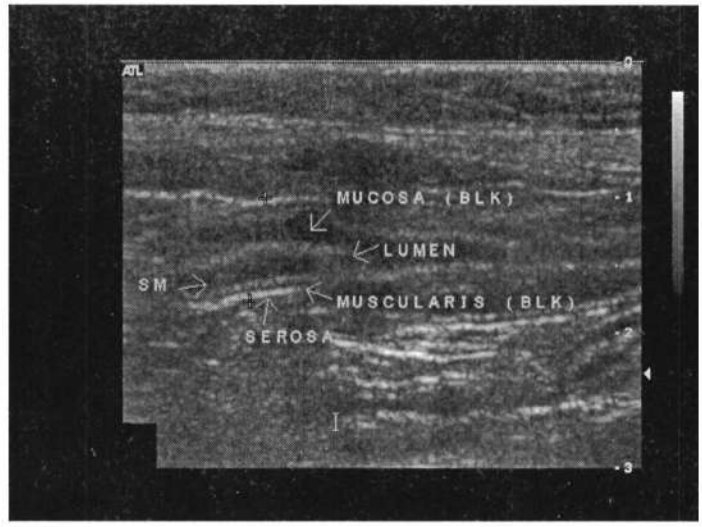
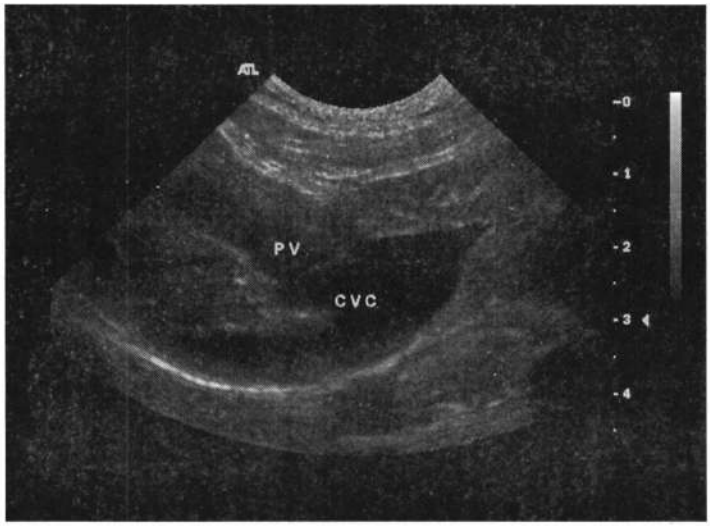


Fig 10 The normal small bowel wall layers.



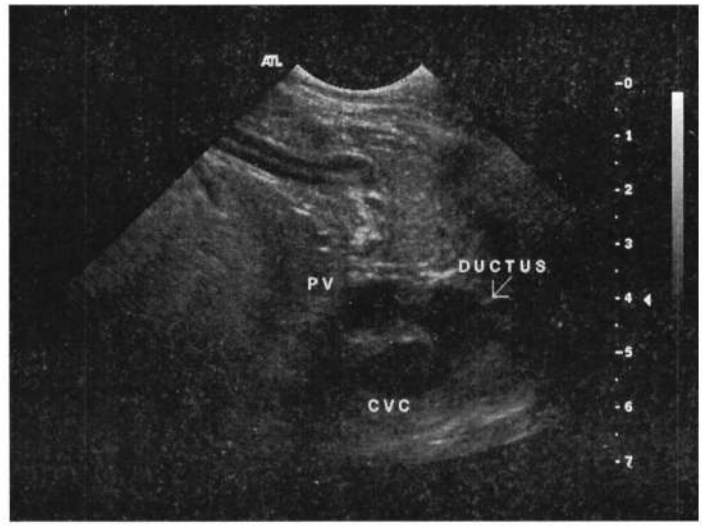


Fig 11, 12 Portocaval shunt (PCS). Sagittal image shows ducting of the portal vein (PV) to the caudal vena cava (CVC); an intrahepatic PCS.

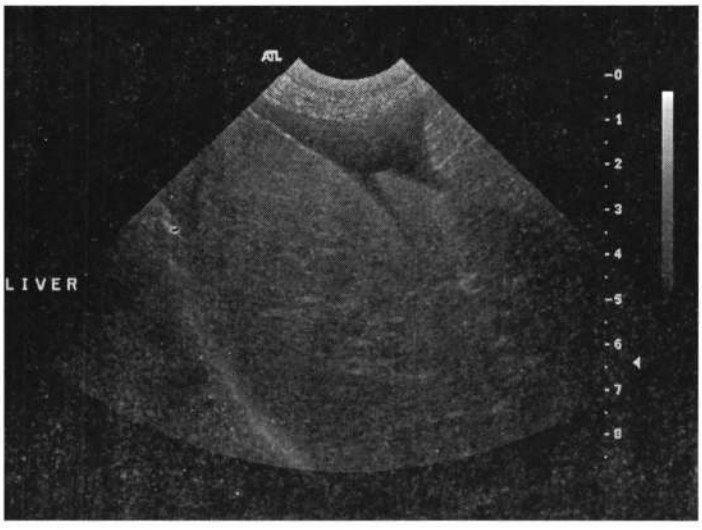


Fig 13 Ascites accenting the border of the liver.

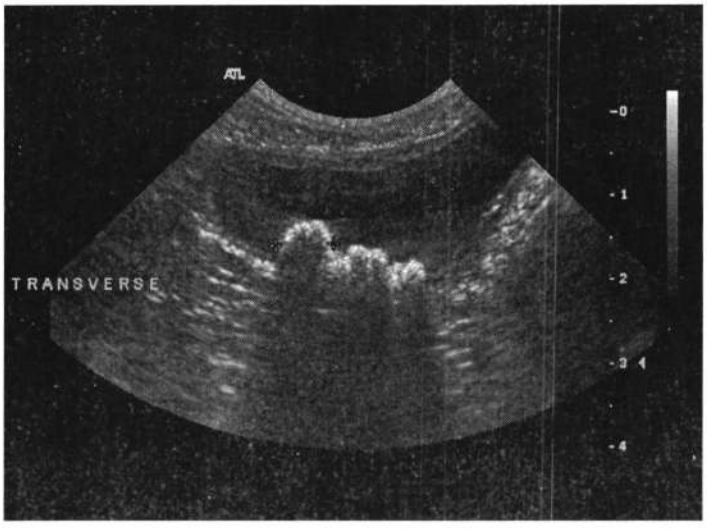


Fig 14 Cystic calculi are visualized along the dependent (dorsal) urinary bladder wall.

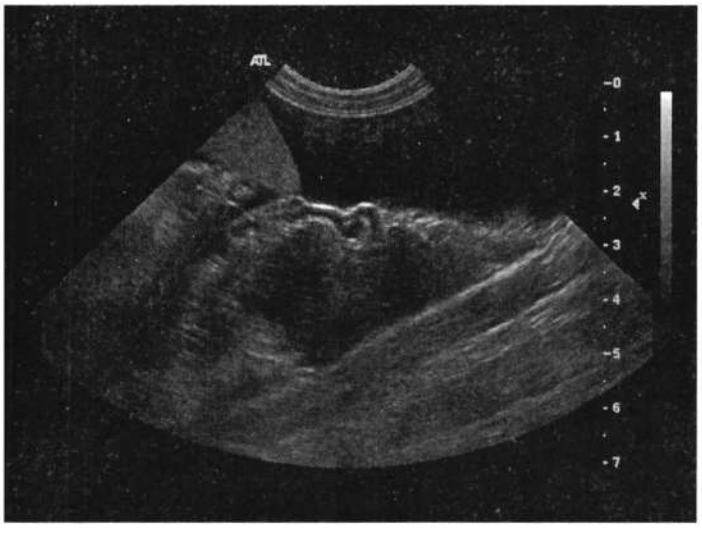


Fig 15 An extrahepatic shunt vessel is seen lateral and dorsal to the spleen. The shunt is tortuous; ascites is present.

Advances in canine semen evaluation techniques

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Abstract

Veterinarians are frequently asked to evaluate dog semen for a variety of reasons, including but not limited to: breeding soundness examination, shipment of fresh, cooled semen, cryopreservation; after a conception failure; after an illness suspect to affect fertility; or after testicular neoplasia or prostatic disease is diagnosed. Ultimately, the goal of any semen evaluation is to predict how likely it is for a male to be successful when used in a breeding situation. Standard semen evaluation in the dog includes determination of semen volume, spermatozoal motility (both total and progressive), velocity, concentration, total spermatozoa/ejaculate, and morphology. Semen morphology during a typical breeding soundness examination is typically performed using one of two stains: Wright-Giemsa or eosin-nigrosin. In the dog, standards for normal semen parameters include a semen concentration of > 10 million spermatozoa/kg bodyweight; >70% progressively motile sperm, and >70% morphologically normal spermatozoa. Many times, semen quality will exceed these parameters yet fertility of the dog may still be suboptimal. If infection and prostatic disease can be ruled out as causes of the subfertility, the clinician is left with attempting to further evaluate the ejaculate to determine the cause of the infertility. Evaluation of the sperm's function is the next logical step.

Male factor infertility is said to account for up to 50% of the failed pregnancy attempts in humans.[1] No estimates have been made for the canine, but clinical experience would tell us that male factor infertility accounts for a significant portion of either non-pregnancy, early embryonic death, or small litter size. When a standard semen evaluation fails to elicit a clear diagnosis of infertility, additional testing of the ejaculate is desirable. This testing may include additional bright field spermatozoal staining techniques which differentiate different parts of the sperm cell; alternate microscopic evaluation of sperm morphology using differential interference contrast or phase contrast morphology; electron microscopy (EM), both scanning and transmission; acrosomal testing; hypo-osmotic swelling testing (HOST); sperm chromatin structure analysis (SCSA); computer assisted spermatozoal analysis (CASA/ASMA); fluorescent antibody staining techniques using flow cytometric analysis; anti-sperm antibody (ASA) testing; assays for reactive oxygen species (ROS); chromosomal studies; and sperm function testing including zona binding assays, sperm penetration assays.

While there is some information on the canine in this regard, much of the information presented will be from human, bovine, and other domestic species research, where this topic has been far more extensively evaluated. The data from other species can be extrapolated to the canine although more research is needed to determine if its use is appropriate in the dog as a predictor of fertility. This paper describes the use of these additional diagnostic methods of spermatozoal analysis for infertility assessment. CASA will only be covered briefly as another paper in this symposium is dedicated to its use.

Keywords: Canine, semen evaluation, male infertility

Introduction

In the human andrology laboratory setting strict criteria have been developed and accepted by the World Health Organization (WHO) for the evaluation of sperm morphology.[1-8] A normal human sperm has a specified size and shape, with a smooth outline, an acrosome that comprises between 40 and 70% of the sperm head, has no neck, midpiece or tail defects, and has no droplets more than ½ the size of the sperm' s head. Use of these strict criteria makes morphologic examination of semen more uniform and allows for easier comparison of research studies. Thus far, no such criteria have been adapted to the domestic species of animals, making semen evaluation less uniform and comparison of infertility studies and application of treatments more difficult.

Bright field microscopy

Bright field microscopy is one of the simplest forms of semen evaluation since every veterinary clinic possesses the equipment to perform the evaluation. There are many different stains which are available for morphologic assessment of spermatozoa. Commonly used stains include eosin-nigrosin (EN), modified Wright-Giemsa, Feulgen, India ink, and Spermac. Each stain has advantages and disadvantages and will be discussed individually.

EN stain has been a conventional stain used for semen morphology in domestic and non-domestic animals for many years.[8,9] Slides stained with EN have a dark background and a white or pink staining spermatozoon. The equatorial ridge can be clearly seen and some defects in the acrosomal cap and sperm head may be identified including knobbed or ruffled acrosomes, diadem defects, and nuclear vacuoles. The midpiece can be differentiated from the tail-piece or flagellum based on the change in thickness between the two structures. Midpiece defects such as thickening, roughening, proximal and distal cytoplasmic droplets, distal midpiece reflexes, coiling and bending

may be determined. Tail defects including bending and coiling as well as duplicity may be diagnosed. EN is also a vital stain. Sperm that have intact membranes and normal chromatin will not allow eosin to be drawn into the cell and so stain white, while sperm with damaged membranes and damaged chromatin will stain pink due to eosin uptake. EN staining overestimates the number of intact membranes when compared to fluorescent assays (see flow cytometry section).[10] Use of live:dead information requires that proper staining procedure is used including prompt staining after collection, prevention of cold shock to the sperm, allowing adequate incubation time before smearing the slide, and rapid drying to prevent cracking or cell death. Round cells cannot be differentiated with this stain. The stain is hypotonic and this may result in stain artifact, such as coiled tails, if they are not dried properly. Cracks in the stain may also be noted if the smear is too thick.

Modified Wright-Giemsa stain (Diff-Quik®; Siemens Healthcare Diagnostics, Deerfield, IL, USA) is a simple, inexpensive, and rapid method of staining sperm.[9] Alterations in head staining may correlate with DNA defects. The sperm head stains deeply basophilic from the equatorial region downward and more lightly basophilic in the area of the acrosomal cap. The midpiece and tail stain eosinophilic. Details of the acrosome are not visible with this stain, so only basic alterations in head size and shape are detectable. Midpiece and tail defects that can be visualized include cytoplasmic droplets, distal midpiece reflexes, bent and coiled midpieces and tails. This stain has the added benefit of allowing differentiation of round cells in the ejaculate, making identification of WBC vs. germ cells possible.

Feulgen staining is a time consuming procedure that allows for better visualization of sperm nuclear and head abnormalities.[9] The DNA in the sperm head stains magenta allowing good nuclear detail and making defects of the nucleus (vacuoles, diadem defects, etc) clearly visible. The acrosome, midpiece and tail do not stain. The stain must be made fresh daily or changes in the pH will affect the intensity of the stain. Slides may be evaluated with either bright field or phase contrast microscopy. The use of phase contrast microscopy results in greater color contrast. Feulgen staining identified more abnormalities of DNA pattern and head shape in the bull sperm nucleus than standard EN stain.[11]

India ink is a one-step stain that is simple and inexpensive to use. It provides a black background and a white or clear sperm outline. It does not allow for good visualization of the acrosome or equatorial region. It does however allow for differentiation of alterations in head size and shape, major defects of the midpiece including cytoplasmic droplets, distal midpiece reflexes, bent or coiled midpieces, and bent or coiled tails.

Spermac® (Conception Technologies, San Diego, CA, USA) is expensive and moderately time-consuming but is technically a simple staining technique.[12] It is important that thin smears of semen are made and are air dried for no more than 10 minutes prior to fixing. Once fixed, the remaining steps in the staining procedure may be delayed indefinitely. The nucleus of the cell stains dark red while the acrosome stains light green, and the midpiece and tail stain dark green. This is an excellent stain for acrosomal evaluation and also allows for good midpiece and tail piece evaluation. Assessment of the nucleus is not as accurate, but head size and shape can be evaluated. Round cells can be visualized but they cannot be differentiated.

Papanicolaou stain is commonly used in human andrology labs, but is not commonly used in veterinary applications due to the complexity of the procedure.[8,13]

Phase contrast microscopy is a form of microscopy that allows small phase shifts of light through a transparent cell which are converted into contrast and amplitude changes in the image.[14] Two light rays are focused exactly inside the opening of the condenser annular ring. The two rays are refracted so that they exit the condenser in parallel. The light is minimally refracted on passing through the specimen and it travels in parallel into the objective where it enters the back focal plane of the objective. A phase plate is positioned in the back focal plane to line up with the condenser annulus.

Differential interference contrast (DIC) microscopy is a system whereby a polarized light source enters a prism and is diverted into two beams at 90 degree angles to each other.[14] These rays pass through a condenser and then the sample. The beams enter and pass through the sample about 0.2μm apart from each other. Since the beams pass through different parts of the cell they follow different optical paths. The beams then enter the objective lens of the microscope and passes through a second prism which recombines the rays into one polarized beam. This recombination leads to interference (since they are on different optical paths) which either brightens or darkens the image. The resultant image appears to be three-dimensional. DIC microscopy provides excellent resolution and clarity of cellular structure with minimal artifacts. It does require that the sperm sample is in a media of similar refractive index to the cells themselves which means that evaluation of semen in skim milk or yolk extenders is difficult.

No staining of the cell is required for either phase or DIC microscopy, but for morphology assessment, the sperm are generally fixed in formol buffered saline and viewed at high power (40 – 100x). Both of these forms of

microscopy provide for more detailed visualization of all parts of the sperm cell including the acrosome, nucleus, midpiece and tail piece than bright field microscopy alone.

Sperm morphologic assessment plays an integral role in predicting success with IUI, IVF and ART techniques.[3-8] Determination of teratozoospermia prior to attempting advanced insemination techniques will help with management choices. For example it has been shown in many human studies that by increasing sperm concentration at the time of insemination, patients with teratozoospermia will have a greater success rate with IUI and IVF procedures.[3-5] In humans it has also been repeatedly demonstrated that once the number of normal sperm drops below 14% infertility is a consistent result; with individuals with 0-4% normal forms have the lowest success rates (45%), 5 – 14% normal forms have moderate success rates (75%), while > 14% normal forms have good success rates (85%) when advanced reproductive techniques like IVF and ICSI are applied.[3-6]

Acrosome stains

Giemsa stain makes the acrosome appear dark purple.[9] It provides good detail of the acrosome but needs to be made fresh for each use and does not allow for evaluation of the sperm nucleus, midpiece or tail.

A sperm triple stain of Trypan blue stains the spermatozoa blue, Bismarck brown stained the post-acrosome region light brown and rose bengal stained the acrosome light red.[15] Sperm can be differentiated into four groups with this stain: dead sperm with fully or partially inactivated acrosomes, dead spermatozoa with missing or degenerated acrosomes, live spermatozoa with reacted acrosomes, and live spermatozoa with active or normal acrosomes. This staining technique has been used for humans, mouse, bull, horse, goat and boar semen.

A one step stain including fast green FCF, rose bengal and ethyl alcohol has been used to stain the acrosome of cat spermatozoa.[16] This stain allows differentiation of acrosome intact, acrosome reacted or damaged sperm, and acrosome non-intact sperm. The slide is examined using bright field microscopy at 1000x.

Coomassie blue stain has been used to assess acrosomal integrity in bulls, boars, and stallions.[17,18] This staining procedure is relatively simple and results in intense blue staining of intact acrosome and lack of stain uptake in acrosome reacted sperm. The results of Coomassie blue staining correlate well with fluorescent staining (see below), DIC and bright field microscopy techniques following incubation with calcium ionophore to induce the acrosome reaction.[17]

Fluorescent stains, like acridine orange, can be used to evaluate sperm that are extenders in opaque extender, like skim milk.[9] Acrosomal integrity can be evaluated using fluorescent microscopy, phase-contrast or DIC microscopy.

Acrosome staining can be performed using bis-benzimide dye Hoescht 33 258 and a FITC-pisum sativum agglutinin (FITC-PSA) after induction of the acrosome reaction by calcium ionophore.[19] Sperm are incubated in TALP media and then calcium ionophore A23 187 is added to induce the acrosome reaction. Then the sperm suspension is permealized in methanol and incubated with a lectin in order to bind the FITC-PSA probe. The Hoescht 33 258 is then used to stain the sperm and fluorescent microscopy is used to differentiate acrosome reacted versus non-acrosome reacted sperm. It is a simple, quick technique to assess acrosome status, but it does not allow for morphologic assessment of the cell itself. Samples in egg yolk based extenders do not hinder using this technique.

Trypan blue or Congo red stain can be precipitated by neutral red and then stained with Giemsa to stain bull, boar and rabbit sperm, but not stallion sperm. This is a simple and reliable staining procedure that results in the stained sperm being classified as live or dead with intact acrosomes, loose or damaged acrosomes, detached acrosome and detached acrosome with no post acrosomal ring.[20]

Hypo-osmotic swelling test

This test is based on the concept that the normal sperm tail membrane will allow fluid to pass into the cell freely under hypo-osmotic conditions. As the fluid flows into the cell, the tail swells. Membrane integrity is important in sperm metabolism and changes in membrane properties must occur for capacitation and the acrosome reaction to occur normally.[8,21] HOST not only assesses the morphologic integrity of the plasmalemma but it also assesses its function and biochemical activity.[8,10] In humans and bulls, there is strong correlation between the HOST, the sperm penetration assay, and there is a good interrelationship between HOST and motility and morphology.[8, 10, 22] In bulls and humans, HOST was a good predictor of success with IVF.[10]

The HOST is simple, fast and inexpensive.[8, 21] One tenth of a milliliter of spermatozoa is incubated in one milliliter of 60 mOsmol fructose solution at 37 °C for 45 minutes. Then 1– 2 drops of this mixture is examined using phase contrast microscopy at 200 x and 400 x. Two hundred sperm are counted and the percentage of sperm with curled or swollen tails is determined. HOST is positively correlated to motility ($r = 0.94$). The premise being motile sperm have normal membranes and will coil or swell when incubated in a hypo-osmotic solution.

As sperm are cooled for increasing amounts of time, decreasing numbers of sperm will be HOS+ indicating damage to the sperm membrane with prolonged cooling.[21] A similar phenomenon is noted after cryopreservation

due to sperm membrane damage. Prolonged heating also damages the sperm membrane resulting in few HOS+ cells. Use of HOST on rewarmed chilled or post-thaw frozen semen may be predictive of the highest quality samples to be used for insemination by selecting for samples with the highest number of HOS+ cells. The HOST may be a beneficial addition to the semen evaluation in dogs with poor fertility but a normal spermiogram.

Acrosome assays, acrosome reaction testing and capacitation testing

To evaluate acrosome status, sperm must be removed from seminal plasma via centrifugation and then are resuspended in capacitation mediim.[23] Hyperactivation can be determined through the use of CASA. The clinical relevance of hyperactivation has yet to be determined. The acrosome reaction can be evaluated through the use of dyes, fluorescent antibodies or lectins. Induction of the acrosome reaction is most readily induced following incubation of the sperm first in capacitation media and then calcium ionophore (A23187) is added.[8,23] It can be added in high concentration and a short incubation period used (30 – 60 minutes) or at low concentration and a long incubation period used (3 hours). The samples are then washed and re-suspended in protein free media and the cells are smeared on slides that are air dried. The slides are then fixed in alcohol and are stained with peanut agglutinin (PNA), Pisum sativum agglutinin (PSA) or fluorescent-labelled lectins and then evaluated with fluorescent microscopy. The number of acrosome reacted cells are then counted and a percentage of all cells is determined.[23,24] Samples that have high numbers of prematurely reacted cells or which do not respond to incubation with calcium ionophore not likely to be able to complete fertilization.[6,7,23,25,26] In humans, there is a high predictive power of induced acrosome reaction and successful IVF outcome.[26]

Acrosome reaction can also be detected using staining with fluorescein-conjugated lectins, like PSA or PNA, plus fluoresceinisothiocyanate (FITC).[8,25] This combination of stains evaluates damage to the acrosome while at the same time differentiating acrosome reacted from acrosome intact sperm. PSA binds to the acrosomal contents while PNA binds to the outer acrosomal membrane.[27] Acrosomal integrity of canine sperm has been successfully assessed using flow cytometry and staining with FITC conjugated PSA and PI.[28] Capacitation status of chilled and frozen thawed canine sperm has been assessed with a chlortetracycline (CTC) assay and CASA for evidence of hyperactivation.[29] Dog semen has been evaluated for its cryopreservability by first inducing the acrosome reaction with calcium ionophore and then staining the sperm with FITC-PNA along with the membrane impermeable DNA supravital stain ethidium homodimer1 (EthD-1).[30] Samples were evaluated with fluorescence microscopy and flow cytometry. The number of cells that underwent the acrosome reaction via ionophore was well correlated with a similar percentage of cells that had acrosome damage post cryopreservation. Furthermore, the amount of damage to cells caused by acrosome reaction from calcium ionophore was strongly negatively correlated with the number of motile sperm present after freezing.

Bovine sperm have been incubated with calcium ionophore to stimulate the acrosome reaction and then fixed in formaldehyde.[31] Afterwards they are stained with naphthol yellow S plus erythrosin B or with naphthol yellow S plus aniline blue. This is a permanent fixative and the use of DIC microscopy is required to evaluate acrosomal status. Alternatively, bovine sperm may be treated with fluoresceinated PSA to assess the acrosome reaction similar to that described previously.[27]

Triple staining techniques for acrosomal evaluation have also been described but are more time consuming than the above mentioned techniques and so are not routinely used in the clinical or research setting.[8]

Sperm penetration assays

These assays asses the ability of the sperm to undergo capacitation, the acrosome reaction, membrane fusion and chromatin decondensation in the presence of an oocyte.[4,6-8,23,32] Sperm must be prepared for the assay by incubating overnight in a capacitation medium or storing in a TES-tris buffer with egg yolk for 24 – 48 hours and then applying thermal shock. After this processing step, the sperm are divided into microdrops and zona-free hamster eggs are added. They incubate for 3 hours and the number of eggs penetrated and the number of sperm/egg are counted. The count is performed by looking for swollen heads within the unstained eggs using phase-contrast or phase-interference microscopy or after staining with acridine orange (AO) and using fluorescence microscopy. Use of the TES-tris buffer procedure was more highly correlated with fertility and successful outcomes with IVF in humans in some studies[4,6,7,23] while in others it's predictive power was questionable.[26]

Hemizona assays

This test assesses the availability of the proper molecules on the sperm's head for it to bind to the zona pellucida and initiate interaction with the oocyte.[8,23] A bisected zona pellucida from a normal oocyte is used. Each half of the zona is incubated with sperm for 4 hours and then the number of bound cells is counted. Zona pellucida binding assays have been used to evaluate the fertilizing capacity of chilled and frozen-thawed canine spermatozoa.[33] The test allows an estimation of the damage caused by manipulation of semen on the fertilizing ability of sperm. This test also demonstrates the critical interaction between the zona pellucida and the sperm cell during fertilization and tests multiple sperm functions, including capacitation and ligand-induced acrosome

reaction.[4,6-8,34,35] Of the classic sperm parameters, morphology was the best predictor of the ability of sperm to bind to the zona pellucida.[3,4,6,32,34] In conventional IVF studies in humans, defective sperm-zona binding and zona penetration are common causes of failure of fertilization.[7,35] There is a high predictive power of sperm-zona pellucida binding and successful outcome with IVF in humans.[26,35]

Electron microscopy – transmission (TEM)

The sperm rich fraction is mixed 1:2 with cacodylate-buffered 6% glutaraldehyde.[8,36] This mixture is centrifuged, the supernatant removed and the pellet resuspended in 0.1 M sodium cacodylate buffer. This sample is washed a second time and the supernatant removed. The pellet is fixed in a solution of 1% osmium tetroxide in 0.1 M cacodylate buffer and is centrifuged. The osmicated pellet is dehydrated through a graded series of ethanol, is then rinsed in propylene oxide and is then embedded in Poly/Bed 812 or araldite. Sections are cut at 80 nm thickness and are then mounted on 300-mesh nickel grids and stained with uranyl acetate and lead citrate for TEM.

TEM may identify lesions of the plasma membrane, acrosome, mitochondria, and nuclear chromatin.[8,36] Quantification of morphologic defects is not possible but a detailed description of the defects is provided. TEM may also help identify and characterize other cells in the ejaculate including germ cells, WBC and infectious organisms. DNA fragmentation caused by oxidative stress or exposure to toxins may be identified.

TEM may identify defects of the tail in patients with motility issues that are not apparent with light microscopy.[8,37] If a single defect is present in at least 20 – 30 sections it is considered a ciliary dyskinetic condition. Total or partial dyein arm deficiency occurs in 3% of human patients with abnormal motility. Fragmentation of the plasma membrane and necrosis of the microtubules is typical of necrospermia and is found in 23% of human patients with asthenozoospermia.[38] Multiple fine ultrastructural defects are noted in another 23% of human patients with this condition. Missing outer microtubules, disorganized axonemes, missing central microtubules, additional microtubules above and beyond 9 + 2, thickened and/or disorganized fibrous sheaths, absent radial spokes and translocated tubules are other common defects noted in this group. Some of these defects are noted alone and others in combination. In patients whose total motility is > 30% and at least some normal tails were evident, pregnancies using assisted reproductive techniques (ART) may be successfully obtained.[37]

A microtubular mass defect was noted on examination of spermatozoa from seven stallions with three of these stallions descending from a single sire.[38] There was subfertility in four of these stallions, although it appeared to be at least partially compensable. Detailed description of the ultrastructure of the bovine sperm head and midpiece are available.[39,40]

Electron microscopy is currently available for clinical cases at the veterinary colleges of Auburn University, Texas A & M University, and University of Saskatchewan.

Antisperm antibody assay

In humans, antisperm antibody production is a common cause of male factor infertility.[8,23] There are two commercial assays (SpermMar®, Conception Technologies, and Immunobead Test®, Irvine Scientific, Santa Ana, CA, USA) available for human antisperm antibody assay. These tests provide semiquantitative results regarding the degree of antibody binding present and detect the presence of IgA and IgG antisperm antibodies. If ≥ 20% of the sperm bind to the beads, a sample is considered positive for the presence of antisperm antibodies.[23] Serum may be assayed for antibodies using a tray agglutination test. At this time, the importance of antisperm antibodies in domestic animals is unclear but may be a useful test for dogs with autoimmune orchitis/epididymitis.

Flow cytometry for DNA and morphology measurement

Binding of fluorescent dyes to sperm chromatin permits the identification of sperm DNA abnormalities and can be measured using a flow cytometer.[8,41] Sperm are stained using fluorescent assay and then run through the flow cytometer to differentiate cells with normal DNA integrity from abnormal. Sperm are typically oriented to be in the same plane before they are excited by a laser beam to induce fluorescence and then flow past a fluorescence detector which monitors exactly how much fluorescence each cell has. Sperm that have uniform head size and shape display a uniform degree of fluorescence while cells with abnormal size and shape have amounts of fluorescence outside the normal ranges.[41] In addition to being able to differentiate morphologically normal from abnormal sperm, flow cytometry can also differentiate sperm with normal motility from those with decreased motility.[42] One cause of decreased motility in humans is a result of a break in the DNA strands of the sperm nucleus and their mitochondria. Flow cytometry and TUNEL (terminal deoxy-nucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling) testing both confirm the presence of these strand breaks in this population of asthenozoospermic men.

One of the greatest assets of using flow cytometry for sperm evaluation is the sheer number of sperm that can be evaluated in a short period of time. Routine microscopic assessment of sperm involves counting either 100 or preferably 200 cells. But with the use of flow cytometry, thousands of cells can be assessed in a matter of minutes.[41]

Another assessment that can be performed using flow cytometry is that of sperm viability. A dual staining technique using carboxifluorescein diacetate (CFDA) and propidium iodide (PI) was used to validate the ability to differentiate live canine sperm from dead sperm.[43] Plasma membrane integrity was also validated in stallion sperm using either CFDA and PI or SYBR-14 stain and PI.[25] In these tests, live sperm fluoresce green (from CFDA or SYBR-14), dead sperm fluoresce red (PI), while dying sperm fluoresce both colors.[25,32]

Tests of mitochondrial activity

Rhodamine 123 (Rh123) is a mitochondrial probe which is combined with the viability stains of PI and carboxydimethylfluorescein diacetate (CDMFDA) to allow for the determination of sperm with intact membranes (CDMFDA+) and functional mitochondria (Rh123+) from dead sperm (PI+).[25] Stallion sperm with functional membranes and mitochondria correlate well with sperm viability and motility.[25]

Cytochemical tests for sperm chromatin integrity

The integrity of nuclear chromatin results from a combination of factors including sperm maturation processes, damage due to oxidative stress and other endogenous factors.[8,44] Chromatin maturation depends on proper replacement of histone with transition proteins and finally basic protamines. This transition results in compact packaging of the DNA and is enforced by cross-linking with protamine-disulfide bonds. In some abnormal sperm, histones may be partially or completely replaced by protamines resulting in loosely packed chromatin. Detection of this loose packing may be found with the aniline blue (AB) test. DNA is more predisposed to denaturation by heat and low pH when chromatin is packed loosely than when it is tightly packed.[44] Chromatin proteins in sperm with abnormal DNA are more susceptible to staining with acidic dyes like AB, acridine orange and toluidine blue.[44] All three of these staining protocols provide a good estimation of the number of sperm with normal vs loosely packed chromatin structure. If more than 30% of sperm have loosely packed chromatin structure an association with increased infertility is noted which correlates with results of SCSA (see below).

Sperm chromatin analysis

Sperm morphology is well correlated with semen quality.[4,7,8,45,46] The head of the sperm consists primarily of nuclear chromatin, so subtle changes in sperm head morphology may be related to abnormalities of DNA content. Measurement of a set of parameters regarding the sperm head (size and shape) and midpiece can align sperm into certain populations of sperm, such that the chromatin content in each sperm head can be determined to be normal or abnormal using automated sperm morphometric analysis (ASMA). Non-compensable defects (those that cannot be overcome by increasing the number of sperm in a breeding dose) are typically related to sperm with abnormal head morphology.[4,7,45,46] Sperm head shape has been correlated with fertility and resistance to cryopreservation. Sperm nuclear DNA fragmentation is positively correlated with lower IVF fertilization rates, impaired implantation, increased risk of abortion, and increased risk of disease in offspring, including pediatric cancer.[7,8,31,47-49,51] Fertile sperm have stable DNA which is able to decondense at the appropriate time during the fertilization process such that the oocyte has access to this DNA for combination with its own DNA complement.[48,49,52]

Sperm DNA damage may occur on several different levels.[4,7,8,31,48- 50,52] Mitochondrial DNA damage can occur and be manifest as deletions, point mutations and polymorphism and is associated with decreased semen quality, asthenozoospermia and male infertility. Nuclear DNA damage may occur as a result of oxidative stress, sperm chromatin packaging and apoptosis. DNA damage may occur as a result of environmental factors, pollutants, infection, inflammation, or the presence of ROS.[48,49]

For SCSA, sperm are treated with an acidic solution (pH1.2) in order to denature their DNA in situ.[8,46,48] Sperm with normal chromatin will not denature under these conditions while abnormal DNA will denature. The sperm are then stained with acridine orange. This is a metachromatic DNA stain. Chromatin which has been denatured into single stranded DNA will fluoresce red, while DNA which does not denature (remains double stranded) will fluoresce green. The percentage of cells with denatured DNA is determined and is called %COMP. The percentage of sperm with non-detectable vs detectable DNA fragmentation is called the DNA fragmentation index (DFI). The percentage of sperm with immature chromatin is called high DNA stainability (HDS). Combining the use of SCSA and ASMA may assist in evaluating dogs with normal spermiograms and poor fertility.[46,48]

DNA fragmentation may also be evaluated using alkaline single-cell gel electrophoresis testing, TUNEL assay; Comet assay, in situ nick translation, and DNA breakage detection-fluorescent in situ hybridization assay (DBD-FISH). These assays use fluorescence microscopy.[7,48-50,52] Staining techniques using aniline blue, toluidine blue and chromomycin A3 also may be used to identify chromatin packaging defects.[52] At this time, few of these assays beyond SCSA are used in clinical practice as it remains to be determined what the clinical relevance of negative outcomes means to fertility. There also still remains significant variability in techniques between labs resulting in disparate results. Certainly in veterinary medicine, data regarding most of these tests in clinical practice is lacking, although they are slowly being introduced.[32]

On the other hand, SCSA, has been accepted as an important tool in the diagnosis of infertility and in prognosticating human couple's success rates with ART.[48,49] In humans, DFI is a strong indicator of successful pregnancy outcome.[4,7,48-50] Prediction of successful outcome with intrauterine insemination is well correlated with the degree of DNA fragmentation.[48,49] The number of sperm that have DNA strand breaks is negatively correlated with their ability to fertilize an oocyte during IVF.[48-50] Sperm DFI was shown to be negatively correlated with sperm concentration, motility and normal morphology.[50] Fertilization failure of sperm with DNA fragmentation may be able to be overcome by application of ART procedures, such as intracytoplasmic sperm injection (ICSI), however embryo development may be affected with resultant early embryonic death (EED) or abortion.[4,7,32,48-52] In humans, a DNA fragmentation rates of > 30% seem to impede fertility, and in couples with high DFI, the use of ICSI will improve pregnancy rates over the use of IVF.[48,49,52]

In stallions, SCSA has been evaluated and shown to be an indicator of some forms of subfertility or infertility.[25,53] Subfertile stallions had higher %COMP levels than did stallions with normal fertility and there was a negative correlation between seasonal pregnancy rate and %COMP, % morphologically normal sperm, and % motile sperm. In boars, there is an inverse relationship between farrowing rate and numbers of pigs/litter compared to %DFI.[48] Pregnancy rates in bulls with high %DFI were lower than for bulls with low %DFI.[48] It appears that the threshold for fertility in bulls (10 – 20%) and boars (8%) is much lower than for humans).[48] A threshold level is not yet available for dogs.

SCSA is currently offered for clinical cases at SCSA Inc (Brookings, SD; www.scsa.com) and at Texas A & M University College of Veterinary Medicine.

Assays for reactive oxygen species

ROS are very important during the sperm capacitation process in order for tyrosine phosphorylation events to occur normally.[7,8] They are normally produced at low levels, however, in some cases of infertility they are produced in much higher amounts. ROS interrupt sperm function by causing peroxidative damage to the plasma membrane and thereby impairing motility, the acrosome reaction (exocytosis), and disrupting sperm-oocyte fusion. Oxidative stress may also cause mitochondrial DNA and nuclear genome damage.[7] There are chemluminescent assays using redox-sensitive probes (lucigenin and luminol) for human spermatozoa.[7,8] High levels of chemluminesence affect the fertilizing capacity of sperm both in vivo and vitro. The presence of WBCs in the ejaculate will greatly increase the amount of ROS present, therefore WBCs must be removed from the samples prior to testing.[7] In the presence of seminal plasma, protection from ROS produced by WBC is afforded, while for ART technologies, these ROS will likely have a much more significant role.

Fluorescence in situ hybridization (FISH)

This procedure allows for the analysis of chromosome numbers in individual sperm. Individuals with oligoasthenoteratozoospermia are at increased risk of having chromosomal abnormalities such as aneuploidy, double aneuploidy and diploidy.[1,54] Sperm are fixed in a methanol:acetic acid solution and then the DNA is decondensed in an acidic salt solution. Sperm probes for specific chromosomes each fluoresce in specific color ranges are applied and in situ hybridization is performed. In humans, teratozoospermia in the form of macrocephalic, multi-tailed sperm have an increased incidence of aneuploidy.[54] Other morphologic abnormalities may also be associated with specific chromosomal abnormalities and this area bears the need for further investigation.[54]

Computer assisted sperm analysis and automated sperm morphometric analysis

CASA is a technique employing a computerized system that tracks mean percentage of motile sperm, mean percentage of progressively motile sperm, mean curvilinear velocity and mean straight line velocity per microscopic field.[8] The ejaculate is diluted to a specified concentration and the system uses a special gridded microscope slide that accepts a constant volume of semen thus providing a consistent number of sperm to be evaluated each time. The pre-warmed and loaded slide is placed into a thermostatically controlled chamber for analysis. The computer takes video images of the sperm and stores then for analysis. The system recognizes motile from non-motile sperm and other organic debris by comparing luminosity (gray-scale intensity) and size of the object. There are also preset user-defined thresholds for size and luminosity that help prevent mistaking other cells and debris for non-motile sperm. Computerized systems have been shown to be more accurate than subjective assessment of sperm motility in human, equine, bovine and canine studies.[6,8,32,55-57] CASA provides a more discriminating estimation of motility than subjective evaluation with greater repeatability.

ASMA is now also available and provides a more accurate and repeatable evaluation of general sperm morphology.[4,6,25,32,46,58,59] With this automated process, as with CASA, sperm are diluted in physiologic media to a specified concentration and then a fixed drop placed on a slide and air dried. The slides are then stained and coverslips permanently affixed to the slide before processing in the analyzer. In this way a consistent number of sperm/field may be analyzed. Staining method is also important. Papanicolaou stain and Giemsa stain have been used successfully for morphometric analysis in humans, stallions and dogs. The machine obtains a variety of head

measurements including length, width, area, perimeter and width/length. A specified gray scale is required and allows differentiation of sperm heads from other cells and debris on the slide. Midpiece width and area, distance between the major axes of the head and midpiece, angle of divergence of the midpiece from the head axis can also be assessed. Abnormal sperm head, midpiece and tail morphology can be detected 95% of the time with these measurements.[45,46,58]

In the normal dog, a significant variation in head area, length, width and roundness exists, but the analyzer still provides accurate differentiation of teratozoospermic samples.[45,46,59] Ovalness was the least variable factor obtained while length and width had more variation between dogs. Within dogs, there was less variation of any measurement. Similar variability in sperm head shape and size is noted with SCSA analysis of the same dogs, indicating that ASMA may be a valuable tool when assessing sperm for teratozoospermia.[46]

In vitro fertilization

This is the ultimate test of sperm function.[8,23] In human medicine, the end point of IVF is what percent of MII oocytes are fertilized and develop to the 8 cell stage by day 3 post insemination.[4,32]

Sperm function tests

Table 1. Sperm functions and the sperm function tests that assess them[23]

Motility	CASA	Light microscopy		
Morphology	ASMA	Light microscopy	EM	Flow cytometry
Capacitation	IVF	SPA		
Acrosome Reaction	IVF	SPA	Acrosome reaction tests	Acrosin assays
Zona pellucida binding	IVF	HZA		
Zona pellucida penetration	IVF			
Oocyte-sperm fusion	IVF	SPA		

CASA – computer assisted sperm analysis; ASMA – automated sperm morphometric analysis; EM – Electron microscopy; SPA – sperm penetration assay; IVF – in vitro fertilization; HZA – hemi-zona assay

Summary

It is clear that there is much more to be evaluated regarding male fertility than the basic semen evaluation. The concept that simply providing a specified number of normal appearing, motile sperm at the proper time in relation to breeding will result in acceptable pregnancy rates is clearly a misconception. There are many aspects of sperm function that may affect the functional competence of the sperm cell, beyond its basic size, shape and motility.[7] When faced with a dog that has subfertility, the clinician must first rule out the most obvious causes for the problem and them move on to more advanced semen diagnostic testing to exhaust all possible diagnoses. In the process of making the diagnosis, the clinician may discover a method of treating or correcting for the problem. Unfortunately, in some cases, even with exhaustive testing, a diagnosis may remain elusive. Research in all areas discussed in this paper is needed for all domestic animals including the canine.

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Synchronization of estrus and ovulation: a practitioner's perspective

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Abstract

Artificial insemination as a management tool can be facilitated by synchronization of estrus or ovulation. Although estrus detection will remain an important part of successful breeding programs, timed artificial insemination is increasingly used in cattle herds. The practicing theriogenologist must utilize his or her knowledge of the physiology of the bovine estrous cycle and reproductive pharmacology, consider constraints imposed by availability of labor and facilities, and choose among the available protocols the one which is most likely to be successfully implemented. This article reviews the physiology of the bovine estrous cycle, and some of the protocols currently in use.

Keywords: Bovine estrous cycle, estrus synchronization, artificial insemination

Introduction

Artificial insemination as a management tool has been extensively utilized by dairy producers for over 50 years, and use in beef cattle has become increasingly common over the last 2 decades. Heat detection and insemination based on estrus signs and behavior remain necessary components for most successful artificial insemination programs, but the veterinary practitioner is increasingly called upon to suggest and monitor protocols aimed at increasing efficiency or convenience of artificial insemination programs by synchronizing estrus or ovulation. Knowledge of the physiology of the estrous cycle, reproductive pharmacology, and management of cattle and people are all necessary components of successful programs.

Estrous cycle control has developed in distinct phases, as the events of the estrous cycle have been understood and pharmacologic agents have become available. Today, the most successful protocols combine control of the length of the luteal phase with control of the emergence of follicular waves, often with control of the time of ovulation and timed artificial insemination. An understanding of the physiology of the estrous cycle, including emergence of follicular waves, development of the dominant follicle, follicular atresia, ovulation, development of the corpus luteum (CL), and luteolysis is a necessary prerequisite to evaluate and implement synchronization strategies.

Integration of the physiology of the estrous cycle with reproductive pharmacology, the age, breed and lactation status of the targeted group, the animal handling skills and facilities present on the farm or ranch, the availability of sufficient skilled personnel at critical times, effective communication and record keeping are all important to success of protocols. The practicing theriogenologist must take all of these into consideration as he or she consults with their clients.

A brief outline of critical points is presented below, and discussion of the some of the more common protocols which have evolved follows.

The bovine estrous cycle

Cattle are reproductively non-seasonal, polyestrous, monovulatory, with an interestrus interval ranging from 18-23 days. Observable estrus behavior is detectable even in the absence of the male.

Heat, or estrus, is best demonstrated by standing to be mounted, whether by a bull or by other females in the herd. Typical estrus behavior is triggered by rising estradiol in an environment where progesterone concentrations are falling.

The rising concentrations of circulating estradiol accompanying estrus induce the leutinizing hormone (LH) surge, and the LH surge induces ovulation. Ovulation follows the onset standing heat by 24 to 32 hours.

Ovulation is followed by development of the CL, a transient endocrine organ that arises on the ovary at the site of ovulation. The CL produces a number of endocrine products, the most important for this discussion being progesterone, which is necessary for maintenance of pregnancy following insemination. Should pregnancy not occur, the CL will regress and another ovulatory follicle will develop. This follicle will produce sufficient estradiol to again induce psychic estrus and the LH surge, and ovulation will follow. The cycle will repeat indefinitely in the absence of pregnancy. This very basic outline of the events of the estrous cycle has proven to be sufficient for insemination programs based on heat detection. As we move to more precisely control the estrous cycle, an understanding of other, less obvious events becomes necessary.

The cycle is sometimes conveniently divided into the luteal and follicular phases, based on the dominant ovarian structure present. The follicular phase begins with the regression of the CL and encompasses about 20% of the cycle, the period from the onset of luteolysis until ovulation. Estradiol from the developing dominant or preovulatory follicle is the predominant sex steroid during the follicular phase. The luteal phase begins at ovulation, and continues until regression of the CL. During this time, the sex steroid dominance shifts to progesterone produced by the CL. The luteal phase includes both metestrus, the period of CL development following ovulation,

and diestrus, the period spanned by the life of the mature CL. Follicles continue to emerge and grow during the luteal phase, and although they produce estradiol, luteal progesterone inhibits LH sufficiently to prevent development to preovulatory size

The endocrine signal that results in regression of the CL in the absence of pregnancy has been known to be prostaglandin F2alpha (PGF2ά) for several decades. More recently, the signal responsible for maternal recognition of pregnancy, which effectively blocks luteolysis, has been shown to be interferon tau produced by the fetal trophoblast.[1] The signals that result in emergence, growth, and regression of ovarian follicles became evident following the advent of ultrasound as a research tool coupled with endocrine assays in the early 1980's.[2] As mechanisms regulating the ovarian cycle were discovered and pharmacologic agents became available, opportunities to develop protocols to control the events of the estrous cycle developed.

Follicular dynamics

During the 1980's, ultrasound technology allowed basic research that described waves of follicular growth and regression. These revelations were coupled with endocrine assays to explain the dynamic nature of ovarian follicles. Understanding of the processes that initiated follicular emergence, selection, atresia, dominance and ultimately ovulation, coupled with strategic administration of the limited number of available pharmacologic agents have become the basis for programs to manipulate the estrous cycle and in many instances precisely control the time of ovulation. The basic research on follicular dynamics has been reviewed extensively in recent publications,[3,4] and the reader is referred to these resources for a more detailed description. A brief summary, applicable to clinical practice follows.

Estrus is characterized by the presence of a large ovarian follicle, producing sufficient estrogen to induce the psychic changes associated with estrus behavior and to induce a surge of LH, which eventually results in ovulation of the follicle and release of the oocyte. Following ovulation, the granulosa and thecal cells surrounding the follicular antrum transform into luteal cells (luteinization) and the CL develops. Steroid production shifts from estrogen to progesterone and this period of progesterone dominance initiates the luteal phase.

At the time of the LH surge which initiates the events of ovulation, both LH and follicle-stimulating hormone (FSH) are released from the pituitary. This release of FSH is followed closely by a second surge of FSH, and these peri-ovulatory surges of FSH are associated with the emergence of a cohort of small follicles from the ovarian follicular pool shortly after ovulation. Emergence, defined as the last day the potential dominant follicle was less than 4 mm, is perhaps more easily understood as the time that follicles are easily detectable with commonly used ultrasound equipment. Emergence of follicles is generally coincident with the peak of the FSH surge. As growth of these follicles is stimulated by FSH, follicular hormonal activity increases and the antral follicles produce a number of endocrine products, including the protein hormone inhibin, and later estradiol.[5] Estradiol and inhibin exert negative influence on the anterior pituitary to decrease or inhibit release of FSH from the anterior pituitary, so that following follicular emergence, FSH concentrations decline. FSH has a long half life relative to LH, but concentrations of FSH remain sufficient to support follicular growth only to a diameter of 8-9 mm,[6] although exogenous FSH administration will allow follicular development to continue past this stage.[7]

Within the cohort of emerged follicles, a single follicle either has or gains a slight developmental advantage as FSH concentrations approach their nadir. As this developmentally advantaged follicle grows, it acquires additional LH receptors in the granulosa cells surrounding the follicular antrum and oocyte.[8] The acquisition of LH receptors is critical for continued development of the follicle, and LH support allows this follicle to continue progressive development as the growth rate of the other follicles in the cohort declines. The time at which the growth rate of the future dominant follicle exceeds the growth rate of the largest dominant follicle is referred to as follicular deviation and the largest follicle is referred to as the dominant follicle. As this selected follicle continues to grow in response to pulsatile release of LH it continues to produce estradiol and inhibin, and FSH support for the remaining follicles is lost. These subordinate follicles, lacking LH receptors in a low FSH environment, begin the process of atresia as the dominant follicle continues to grow. As the dominant follicle grows and produces estradiol, a positive feed back loop with the hypothalamus increases the frequency of gonadotropin-releasing hormone (GnRH) pulses, which in the face of continued inhibition of FSH release, results in increased pulsatile LH, but not FSH, release from the pituitary.

Coincident with the development of this first post ovulatory follicular wave, and eventual development of a single dominant follicle, is the development of the CL and the associated increase in circulating progesterone concentrations. As noted above, the dominant follicle grows and increasingly produces estrogen which feeds back on the hypothalamus to increase GnRH and elicit LH pulses from the pituitary. However, in the high progesterone environment of the luteal phase, GnRH release is limited, and the resultant LH pulsatility is insufficient to support continued development of the dominant follicle to pre-ovulatory size. The dominant follicle, deprived of sufficient LH for continued development, eventually joins the other members of the cohort, and undergoes atresia. Atresia of

the dominant follicle removes the source of estradiol and inhibin, and shortly after its demise, another surge of FSH from the anterior pituitary stimulates emergence of a second follicular wave.[9] The second follicular wave develops in a manner similar to the first, and a new dominant follicle develops. The dominant follicle of the second follicular wave may go on to become the ovulatory follicle, or may undergo atresia and be replaced by a dominant follicle from a third follicular wave which will become the ovulatory follicle.

In the non-pregnant animal, the endometrium releases PGF2ά by day 16 -17 post ovulation, and destruction of the CL (luteolysis) follows. As circulating progesterone concentrations decrease following luteolysis, the inhibitory effect of progesterone on GnRH release is removed. LH pulse frequency increases, and the dominant follicle responds with continued growth. This growth is accompanied by increasing estrogen production, and feedback on the hypothalamus further increasing pulsatile release of GnRH, which it turn acts on the pituitary to increase LH pulse frequency. Follicular development continues in this low progesterone environment with the production of estrogen eventually reaching the threshold necessary to trigger the LH surge.

Most cattle exhibit either 2 or 3 follicular waves during an estrous cycle. It is necessary to recognize that follicles which achieve dominance can only reach pre-ovulatory status in the low progesterone environment that follows luteolysis, and that any of the dominant follicles produced in either 2 or 3 wave cycles can grow to ovulatory size if luteolysis is induced and the inhibitory effect of progesterone removed. Figure 1 is a schematic representation of ovarian follicular development during a typical three wave estrous cycle.

Pharmacologic control of the estrous cycle

Programs to control the time of estrus and ovulation developed as the events controlling various portions of the estrous cycle were recognized, and as products for pharmacologic manipulation of these events became available. Even though the number of pharmacologic agents available is limited, producers and veterinarians are offered an ever increasing number of protocols for control of the estrous cycle, with seemingly endless variations and refinements. Veterinarians actively practicing in the field as theriogenologists are asked and expected to evaluate estrus and ovulation synchronization programs, and make recommendations regarding selection and implementation on specific premises. Understanding the available hormones and their interactions with the events of the estrous cycle are essential in this task.

Pharmacologic agents to control the bovine estrous cycle

Opportunities for manipulation of the estrous cycle in cattle are limited to control the length of the luteal phase, initiation a new follicular wave, and control the time of ovulation. Many of the currently available programs rely on control of all three. Precise control of ovulation has increasingly led to adoption of timed artificial insemination (TAI) protocols.

Drug availability is influenced by legal constraints which prohibit the use of some drugs and classes of drugs. Products must be available from commercial sources, and the use of compounded drugs for estrous cycle control is prohibited by the Animal Medical Drug Use Clarification Act (AMDUCA). Enforcement of restrictions seems likely to increase, driven by food safety and consumer demands.

Available products meeting these criteria and commonly utilized in clinical settings are limited to progesterone and progestins, prostaglandin F2ά (PGF2ά) and its analogs, and GnRH agonists. Other potentially useful products such as FSH, LH, or human chorionic gonadotropin (hCG), are uncommonly utilized in synchronization protocols. Noticeably, and deliberately absent from this list are injectable estrogenic compounds, which although valuable and historically widely used, are not currently approved for estrus control in food animals, and not commercially available in the United States. As mentioned earlier, compounded products, including estrogen, must be avoided. Table 1 provides a list of some commercially available products.

Control of the length of the luteal phase

The length of the luteal phase may be extended by administration of exogenous progesterone or progestins, or truncated by the administration of luteolytic doses of prostaglandin.

Extending the luteal phase with progestins

Early attempts at synchronizing estrus focused on administration of progesterone or progestins, followed by acute withdrawal. In this manner, the period of progesterone dominance is extended beyond the normal lifespan of the corpus luteum. Exogenous progesterone inhibits release of LH, preventing development of ovarian follicles to preovulatory size. Removal of the exogenous source or progesterone from groups of cattle results in a relatively synchronized estrus followed by ovulation. An additional benefit of progestin therapy is the ability to hasten the onset of estrous cyclicity in animals that may be anestrous at the beginning of the protocol.

Melengestrol acetate. Melengestrol acetate (MGA), an orally active progestational steroid, was developed in the 1960s and first marketed to both improve feed efficiency and rate of gain in feedlot heifers and suppress estrus behavior. Suppression of ovulation and estrus behavior occurs when consumption was approximately 0.5 mg per head per day.[10]

Early efforts at synchronization with MGA in cycling cattle relied on feeding 0.5 to 1 mg daily for 14 to 18 days, a period sufficient to allow spontaneous luteal regression in all animals within a group. Because the luteal phase is extended past the time of luteolyis, follicular development and ovulation is suppressed. Following withdrawal of MGA from the diet, follicular development resumes and a synchronized estrus follows. A majority of animals treated in this manner exhibit signs of estrus and ovulate 3 to 7 days following MGA withdrawal. Pregnancy rates to this synchronized estrus are variable and generally disappointing. The reduction in fertility, however, is not apparent in subsequent cycles. Because the reduction in fertility is confined to the first post treatment estrus, programs have developed to take advantage of the synchrony of the second post treatment cycle. These programs are plagued by decreasing synchrony of the second post treatment estrus due to the inherent variability of estrous cycle length. These protocols require that the synchronization be planned well in advance of the onset of the breeding season.

The reduced fertility of the first synchronized estrus following MGA withdrawal has been attributed to altered development of ovarian follicles. Dominant follicles which would either undergo atresia or ovulate during a normal ovarian cycle persist beyond their normal lifespan in the sub-luteal progesterone environment provided by the exogenous MGA. Following withdrawal of the progestin, these persistent follicles grow, produce sufficient estradiol to induce estrus and the LH surge, and ovulate, but the oocytes associated with these follicles are often developmentally compromised.

Administrating MGA in the feed for shorter intervals partially overcomes the negative effects of persistent follicles, but synchrony of estrus is decreased. Incorporating higher doses of MGA has not been effective in overcoming problems associated with development of persistent follicles.[11] Although some reports indicate acute administration of progesterone late in the artificially lengthened luteal phase will induce follicular turnover,[12] the lack of FDA approval for injectable progesterone products precludes use of this strategy.

Several variations of the initial MGA protocol utilizing PGF2ά have been developed to overcome problems associated with lack of synchrony of the second post treatment estrus, and will be discussed in a later section.

Intra-vaginal progesterone. Intra-vaginal delivery of progesterone as a method of extending or controlling the length of the luteal phase of the cycle has been available in the United States since 2002. Currently the only FDA approved device is the Eazi-Breed CIDR® (Pfizer Animal Health, New York, NY, USA). The Eazi-Breed CIDR® has been available for many years in other countries prior to introduction to the U.S. and a large number of clinical and research trials have demonstrated the efficacy of progesterone delivery by this route.

Labeled protocols for Eazi-Breed CIDR® specify that the pessary be placed intra-vaginally for 7 days, with administration of PGF2ά either one day prior to pessary removal, or on the day of removal. The protocols yield similar results.[13] This combination approach allows precise control of the length of the luteal phase, and synchronizes estrus. Because of inherent variations in follicular wave emergence, estrus activity following this protocol is not synchronized sufficiently to permit appointment breeding. The range of estrus activity is often much more tightly controlled than in programs which use prostaglandins as the sole agent.

Progesterone-releasing pessaries are often incorporated into other synchronization programs to improve the degree of control of the luteal phase, and like other progestin based synchronization protocols, may hasten the onset of cyclicity in anovular cattle.[14,15]

Truncating the luteal phase with prostaglandin F2ά

Almost 3 decades ago, PGF2 ά and its analogs were introduced as the first drugs approved to control the estrous cycle in cycling cows. Prostaglandin is released from the endometrium after mid-cycle in non-pregnant cattle, and result in regression of the CL, removing the progesterone mediated inhibition of LH pulsatility. Although the developing CL is resistant to the effects of prostaglandin, sensitivity increases as the CL matures, and by day 6 post-estrus, the luteal phase can be terminated in virtually all cows administered exogenous PGF2ά.

Administration of PGF2ά or its analogs after day 5-6 of the estrous cycle results in CL regression and loss of progesterone dominance. When administered to groups of cycling cattle, the synchronized end of the luteal phase is followed by a synchronized estrus.

Following the commercial introduction, PGF2ά protocols rapidly became popular in estrus synchronization protocols both for artificial insemination and in synchronizing estrous cycles for recipients in embryo transfer programs. The two commercially available products, dinoprost (Lutalyse®; Pfizer Animal Health, New York, NY, USA) and cloprostenol (Estrumate®;Intervet/Schering–Plough animal Health, Summit, NJ, USA), are in similar in their action, and while cloprostenol has a longer duration of action, their effects are clinically comparable.[16]

Following a single injection of PGF2ά to randomly cycling groups of cows or heifers, 60 to 80% can be expected to be in estrus within a few days following luteolysis. Poor to no response is expected in cattle which have ovulated recently. Cattle which have undergone spontaneous luteolysis would typically exhibit estrus coincidentally

with their "synchronized" herd mates. Single injection protocols can be tailored to a producer's needs is several ways.

Traditional estrus detection with artificial insemination for 6 to 7 days, followed by PGF2ά administration to those not previously inseminated increases the percent response by removing those with immature CLs from the pool. Animals injected are expected to exhibit estrus in 2 to 5 days. Drug costs are very low in this protocol, but the number of days during which animals need to be observed and bred is fairly high. These single injection programs are useful and widely practiced in many farm settings, but many management situations exist in which a shorter window of estrus synchronization is desired. In these cases, group synchronization utilizing 2 injections of prostaglandin may offer advantages.[17]

In two injection schemes, animals which respond to the first injection and animals in the group which have undergone spontaneous luteolysis just prior to administration of the drug are expected to be in heat in from 2 to 5 days. Those animals with an immature CL, (~ day 1-5 of the cycle) are not be expected to respond to the injection of PGF2ά, and luteal development will continue. At the time of the second injection, all animals in the group should be an appropriate stage of the luteal phase and expected to exhibit a synchronized estrus.

Although label indications for dinoprost suggest a two injection scheme with the injections separated by 10 to 12 days, 14 day intervals are more commonly used and 14 day programs are considered equally effective if not superior to the shorter intervals specified on the product label.

Strategies aimed at identifying cows at the appropriate stage of the cycle to respond to PGF2ά injections eliminate injections in animals that will not respond to exogenous prostaglandin. Transrectal palpation for the presence of a CL, determination of milk progesterone concentrations,[18] or identification of the CL with ultrasound have been investigated as methods to identify cattle with functional luteal tissue. Although these techniques can be effective, routine administration of PGF2ά to non-inseminated animals at random stages of the estrous cycle has remained the most common, and perhaps most economically justifiable, procedure.

Estrus synchronization protocols which use PGF2ά as the sole agent are characterized by an inherent variability in the time of the onset of estrus, typically exhibiting a bell shaped curve with estrus activity beginning 2 days post injection, peaking in 3 to 4 days, and declining rapidly after day 5. While this synchronization of estrus is useful and advantageous compared to observing and handling cattle over an entire estrous cycle, synchrony of ovulation is too variable to allow timed artificial insemination protocols.

MGA plus prostaglandin F2ά

The availability of PGF2ά products led to estrus synchronization protocols which combined feeding MGA for various lengths of time with injections of prostaglandin. Feeding MGA for 14 to 18 days, as described above, results in synchrony of estrus, but poor fertility is associated with the first estrus following prolonged feeding of the progestational agent. Because estrous cycles in cattle fed MGA are synchronized following withdrawal of the progestin, there is an opportunity to administer PGF2ά to a group of animals with a synchronized luteal phase following MGA withdrawal. Treatment with prostaglandin late in the luteal phase reduces the variability in the interval from PGF2ά injection to estrus. Following treatment with PGF2ά, synchrony of the second estrus following MGA withdrawal is better than in MGA protocols which do not control the luteal phase length prior to the second synchronized estrus, and fertility to the synchronized estrus is not compromised. Initial protocols injected prostaglandins at 17 days after withdrawal of MGA, and more recently protocols in which prostaglandin injection has been delayed until 19 days after MGA withdrawal have shown tighter estrus synchrony.[19] Detection of estrus and breeding based on the signs of estrus are recommended. These combination programs are quite effective and economical in situations where feed intake is adequately controlled. The length of treatment in these MGA protocols remains a disadvantage, requiring a long lead time prior to insemination.

Shorter MGA programs combined with prostaglandin injections at the time of MGA withdrawal either did not overcome the infertility associated with longer feeding periods,[20] or did not show improvement over PGF2ά alone.[21]

Synchronization of the emergence of the follicular wave

Following atresia of the dominant follicle, a new cohort of follicles emerges in response to FSH, and the transition from the dominance of one follicle to emergence of a new cohort of developing ovarian follicles is termed follicular turnover. Treatments aimed at ending the period of follicular dominance will induce follicular turnover, allowing more precise control of the events and timing of the estrous cycle.

At least three methods to induce synchronous emergence of a new follicular wave are practiced, but only one lends itself to mass synchronization schemes.[22] Aspiration or ablation a mid-cycle dominant follicle removes the inhibitory effects of estradiol and inhibin on pituitary FSH release, and is followed by wave emergence in 1 to 2 days. Administration of an acute dose of exogenous estradiol or a combination of estradiol and progesterone results in emergence of a new follicular wave in approximately 3-4 days. Administration of GnRH can induce ovulation or

luteinization of a dominant follicle, removing the source of inhibin and estradiol, followed by emergence of a new follicular wave in 1.5-2 days.[22,23]

Follicular ablation is not practical in on farm settings involving synchronization of the estrous cycle of groups of cattle. As previously noted, the lack of commercial sources of injectable estrogens and the legal atmosphere surrounding the use of compounded pharmaceuticals in food animals effectively removes administration of estrogen or estrogen/progesterone injections as an option for synchronizing follicular wave emergence.

Although not specifically approved by the Food and Drug Administration for this purpose, GnRH is available in the United States and licensed for use in cattle. Administration of GnRH is currently the basis for control of follicular wave emergence and ovulation in many synchronization programs.

Gonadotropin-releasing hormone

GnRH, a decapeptide hormone produced in the hypothalamus and transported to the anterior pituitary, influences the secretion of the hormones FSH and LH. Commercial formulations have been available in the United States and worldwide for nearly 3 decades, and are approved for treatment of cystic ovarian disease. Currently, no meat or milk withdrawal periods are required for use of this product in cattle.

Endogenous GnRH stimulates release of LH and FSH, with a varying magnitude of response and ratio depending on the ovarian structures and resultant hormonal mix present. During the luteal phase of the cycle, high progesterone concentrations limit the release of GnRH, and subsequently LH pulse frequency. Conversely, during the follicular phase, removal of the negative influence of progesterone is followed by continued growth of the dominant follicle, and increasing production of estradiol by the follicle triggers increasing pulsatile release of GnRH from the hypothalamus. This feedback loop culminates in the pre-ovulatory LH surge, which induces ovulation and initiates the onset of another luteal phase.

Exogenous GnRH administered in the luteal phase during the period of follicular dominance results in ovulation or luteinization of dominant follicles, and is followed by emergence of a new follicular wave. Administration shortly after follicular wave emergence is less likely to result in initiation of a new follicular wave. Administration of exogenous GnRH following spontaneous or induced luteolysis can be used to induce an LH surge and control the time of ovulation. The actions of GnRH have been incorporated into synchronization protocols which control the time of follicular wave emergence and ovulation.

Ovsynch and related protocols

In 1995, a protocol for TAI using sequential administration of GnRH, PGF2ά, and GnRH to synchronize follicular wave emergence, luteolysis, and ovulation was introduced.[24] This protocol, widely known as Ovsynch, was the first allowing appointment breeding to gain widespread acceptance. The basic program with numerous modifications is the basis for many of the successful ovulation synchronization protocols used today.

Figure 2a illustrates a timeline schematic for the Ovsynch protocol. Figure 2b outlines an injection scheduling calendar for Ovsynch. On day 0, all eligible cattle receive GnRH, followed 7 days later by PGF2ά. Forty eight hours later, a second injection of GnRH is administered, and TAI, without regard to heat detection, is carried out 16 to 20 hours following the second GnRH. The initial injection of GnRH is intended to synchronize emergence of a new follicular wave following induction of ovulation of a dominant follicle. The corpus luteum formed following this diestrus ovulation will not become susceptible to the luteolytic effects of prostaglandin for several days, and will provide a source of progesterone should the CL which developed following the previous ovulation be destroyed. The prostaglandin injection 7 days after initiation of the protocol will initiate luteolysis and allow continued development of the dominant follicle which arose from the induced follicular wave to pre-ovulatory size. The second injection of GnRH will induce an LH surge, with ovulation following in approximately 28 hours.[24]

Not all randomly cycling cattle have equivalent ovarian structures. Synchrony of ovulation is improved if a dominant follicle is present at the time of the first GnRH injection compared to initiation prior to the time of follicular deviation. The day of the cycle at which Ovsynch is initiated influences pregnancy rates to timed artificial insemination. Cows in which Ovsynch was initiated near mid-cycle had greater rates of synchronous ovulation following the first injection of GnRH, and programs beginning on day 5 to 12 of the estrous cycle resulted in greater pregnancy rates.[25,26]

Many modifications to the initial Ovsynch protocol have been developed with the goal of increasing rate of synchronous ovulation and pregnancy. The most common have utilized 2 prostaglandin injections, approximately 2 weeks apart, with the last injection 12 or 14 days prior to the first GnRH injection of Ovsynch. These programs increase the percentage of animals at the optimum stage of the cycle when the first injection of GnRH is administered, and are collectively referred to as Pre-Synch Ovsynch programs, or Prostaglandin Pre-Synch programs. Pregnancy rates to TAI are improved compared to Ovsynch alone.[27] A sample schedule for a Pre-Synch Ovsynch protocol is shown in Figure 3. The long lead time (initiation of hormone injections 36-38 days prior to insemination) present an obstacle to use of Pre-Synch, which is minimized in dairy herds by initiating the protocol

during the voluntary waiting period during which breeding is commonly withheld for 45 to 70 days following parturition.

More recently, protocols utilizing both GnRH and prostaglandin to increase the percentage of cows that ovulate following the first GnRH injection have been developed with improvement in pregnancy rate following timed artificial insemination similar to or better than that seen with Pre-Synch Ovsynch programs.[28,29] The injection schedule for the G6G protocol described by Bello is illustrated in Figure 4. The injection of PGF2ά which initiates the protocol induces luteolysis of mid- and late-cycle CL's. Two days later, an injection of GnRH synchronizes ovulation following luteolysis, and initiates a new follicular wave in animals which have a dominant follicle. An Ovsynch protocol is initiated six days later. The goal is to optimize the number of animals with a dominant follicle at the onset of Ovsynch, and ultimately synchronization of ovulation at the completion of Ovsynch.[28] The G6G protocol has a shorter lead time than Pre-Synch, but has scheduling disadvantages. Increased pregnancy rates to timed artificial insemination compared to Ovsynch alone are reported.

MGA plus PGF2ά plus GnRH protocols

Estrus synchronization protocols incorporating oral administration of MGA, followed with administration of PGF2ά have been modified by incorporating GnRH near the time of the first post MGA estrus to synchronize the first follicular wave and ovulation.[30-32] These programs generally allow for shorter duration of MGA feeding, and offer the potential advantage of hastening the onset of cyclicity in the late pre-pubertal period or post partum period. Adaptations allowing fixed-time artificial insemination[33] or heat detection are utilized. Figure 5 illustrates a treatment schedule for the MGA 7-11 Synch modification with fixed time insemination.

Synchronization of ovulation and timing of insemination

GnRH administration in the low progesterone environment following luteolysis results in an LH surge followed by ovulation in 24 to 32 hours. Protocols which used estrogen to induce ovulation enjoyed some popularity,[34] but no approved product is available for veterinarians at this time. Agents with LH activity, such as hCG, could be a suitable substitute for GnRH, but protocols utilizing this strategy are not common.

Timing of insemination should include ample opportunity for sperm capacitiation prior to ovulation, and insemination at 16 to 20 hours following the ovulation-inducing dose of GnRH is the most common recommendation.[35] Earlier insemination, including at the time of administration of GnRH (Co-Synch protocol), may result in acceptable pregnancy rates, while insemination later than 24 hours after GnRH leads to less favorable outcomes.

In the original Ovsynch protocol, the second GnRH injection, administered to synchronize ovulation, was given 48 hours following induction of luteolysis with PGF2ά. This timing was convenient for dairies, and resulted in grouping of chores associated with the protocol around times when cows were handled or locked up. Recent studies suggest that delaying the administration of the second GnRH injection until 56 hours after induced luteolysis will improve pregnancy rates.[36] Modification of any protocol should take into consideration farm schedules to insure compliance, particularly if handling or restraint occurs outside the normal daily or weekly routine.

Choosing and implementing the right protocol

Many estrus synchronization and TAI programs are available, and when implemented properly, most are effective. Selecting a protocol for a particular herd will depend on the ability of herd management to comply with the time lines inherent in each protocol. A lack of commitment or cavalier attitude concerning the timing of injections and breeding will negatively impact the ultimate success any program.

An old saying in the dairy business is "When heat detection is everybody's job, it usually means it is nobody's job". This wisdom is applicable to scheduling and administration of protocols. Unless a job is a priority, compliance will suffer.

Accurate record keeping and proper administration of all hormones are essential. Injection schedules can become very complicated when dealing with large herds and when multiple protocols used within a herd. Both PCDART and DAIRYCOMP 305 have routines which will generate action lists to facilitate scheduling of injections.

Timed artificial insemination programs may overcome some management problems associated with estrus detection, but management problems with nutrition, housing, semen handling, and concurrent disease are not eliminated.

Drug cost, availability of trained technicians, class of livestock and the facilities available for repeatedly handling cattle will influence the choice of protocols for individual farms. Selection of the appropriate protocol for a particular herd or management system means selection of the protocol herd management will be most likely to efficiently and consistently implement, rather than the newest or most popular protocol available.

Discussion

Today, theriogenologists have an increasing number of options to synchronize estrus and ovulation. These systems developed in concert with an increased understanding of reproductive endocrinology and the proper

selection and implementation of any of the available protocols requires an understanding of follicular dynamics, luteolysis and ovulation. Programs which allow for the successful utilization of TAI require much more precise control of the events of the estrous cycle than those which incorporate estrus detection. Although the legal and regulatory environment restricts or forbids access to certain potentially useful pharmacologic agents, successful strategies utilizing available agents have been devised, and offer adequate control of the cycle.

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Figure 1.

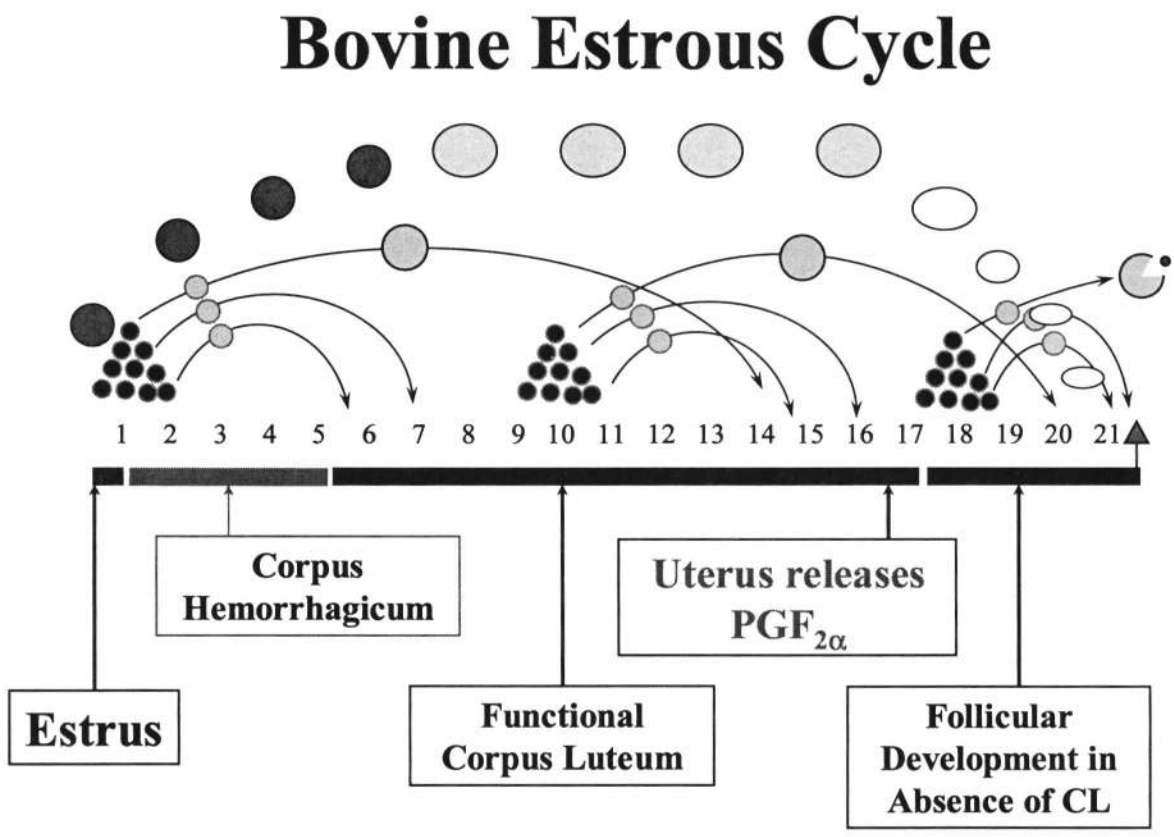

Figure 1: Development of successive waves of ovarian follicles during the estrous cycle. Shortly after ovulation, a cohort of follicles emerges; a single follicle gains an advantage that allows it to become the dominant follicle, only to undergo atresia in the high progesterone environment of the luteal phase. A second follicular wave emerges around day 8-9 of the cycle, and the dominant follicle that follows meets the same fate. A third wave emerges on day 16-17 following demise of the second wave dominant follicle. Prostaglandin-induced luteolysis destroys the source of progesterone, the inhibition of LH pulses is removed, and this third wave dominant follicle develops to preovulatory size. Estradiol produced by the dominant follicle increases past the threshold necessary to induce the LH surge, and ovulation follows. Image courtesy of Dr. M. Daniel Givens, Auburn University

Figure 2a.

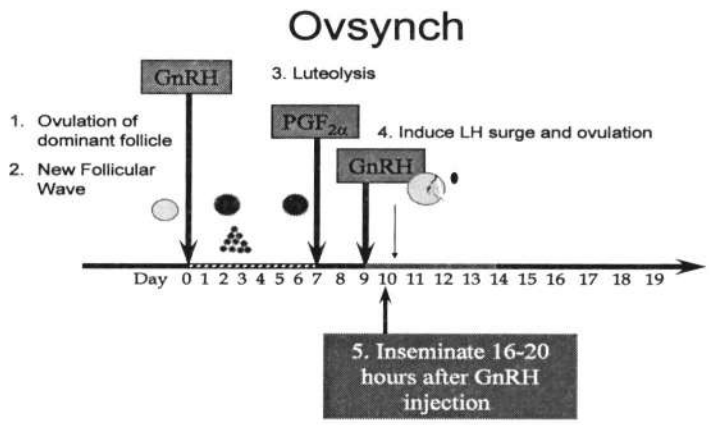

Figure 2a: OVSYNCH PROTOCOL. An injection of GnRH on day 0 induces ovulation or luteinization of a dominant follicle, and a new follicular wave emerges 2 days later. Injection of prostaglandin F2alpha on day 7 induces luteolysis, and injection GnRH on day 9 induces an LH surge, with ovulation 28 hours later. Insemination at 16-20 hours after the second GnRH injection (~8 hours ahead of ovulation) allows time for sperm capacitation prior to ovulation.

Figure 2b.

	SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SAT
Week 1		GnRH					
Week 2		PGF2ά		GnRH	TAI (16-20 hours post GnRH)		

Figure 2b: Injection calendar for a Pre-Synch Ovsynch protocol that avoids weekend chores.
PGF2ά = prostaglandin F2alpha, GnRH = gonadotropin-releasing hormone, TAI = timed artificial insemination.

Figure 3.

	SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SAT
Week 1		PGF2ά					
Week 2							
Week 3		PGF2ά					
Week 4							
Week 5		GnRH					
Week 6		PGF2ά		GnRH	TAI (16-20 hours post GnRH)		

Figure 3: Injection calendar for a Pre-Synch Ovsynch protocol which utilizes 2 prostaglandin injections, 14 days apart, with the second injection given 14 days prior to the start of Ovsynch. This protocol minimizes the number of days per week on which injections are given, avoids weekend chores, and places all prostaglandin injection on the same day of the week. A modification in which the first two prostaglandin injections are given on Wednesday of week 1 and 3 could be utilized and may have advantages.
PGF2ά = prostaglandin F2alpha, GnRH = gonadotropin-releasing hormone, TAI = Ttmed artificial insemination.

Figure 4.

	SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SAT
Week 1		PGF2ά		GnRH			
Week 2			GnRH				
Week 3			PGF2ά		GnRH	TAI (16-20 hours post GnRH)	

Figure 4: Injection calendar for a modified G6G protocol. G6G shortens the lead time prior to insemination compared to Pre-Synch Ovsynch, but has the disadvantage of having assigned chores related to the protocol on more days of the week. Scheduling to avoid weekend chores may be more complicated with this protocol.
PGF2ά = prostaglandin F2alpha, GnRH = gonadotropin-releasing hormone, TAI = timed artificial insemination.

Figure 5.

	SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SAT
Week 1						MGA (first day)	MGA
Week 2	MGA	MGA	MGA	MGA	MGA (last day) PGF2ά		
Week 3		GnRH					
Week 4		PGF2ά		GnRH and TAI (60 hours after PGF2ά)			
Week 5							
Week 6							

Figure 5: Treatment calendar for MGA 7-11 Synch with TAI. MGA feeding begins on day 0, and is discontinued on day 7. Prostaglandin injections are given on day 7, and followed on day 11 with GnRH injections. On day 18, prostaglandin is injected to end the luteal phase, and GnRH is injected and cows are inseminated at 60 hours following the prostaglandin injection. Variations eliminating the last GnRH injection utilize heat detection and breeding based on the signs of estrus.
MGA = melengestrol acetate incorporated into feed, PGF2ά = prostaglandin F2alpha, GnRH = gonadotropin-releasing hormone, TAI = timed artificial insemination.

Table 1.

Class	Drug	Trade Name	Route of Adminstration	Manufacturer
Progestatational agents	Melengestrol acetate	MGA®	Oral	Pfizer
	Progesterone	Eazi-Breed CIDR®	Intravaginal pessary	Pfizer
Prostaglandin F2α and analogs	Dinoprost tromethamine	Lutalyse®	Intramuscular injection	Pfizer
	Cloprostonol	Estrumate ®	Intramuscular injection	
GnRH agonists	Gonadorelin diacetate tetrhydrate	Cystorelin®	Intramuscular injection	Merial
		Ovacyst®	Intramuscular injection	
	Gonadorelin hydrochloride	Factrel®	Intramuscular injection	Fort Dodge Animal Health

Table 1: Commercially available products used to manipulate the estrous cycle of cattle.

Antimicrobial therapy in bovine reproduction

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Abstract

The use of antimicrobials, both systemically and locally, has been a hallmark of therapy in the treatment of uterine infections in cattle. However, the use of antimicrobials has not been without controversy regarding their efficacy, effects on future fertility, risk for bacterial resistance and potential residues. This article reviews the immunology and pathophysiology of postpartum uterine infections in cattle and evaluates research regarding the use and efficacy of local and systemic antimicrobial therapies.

Keywords: Bovine uterus, metritis, endometritis, antibiotic therapy

Introduction

The use of antimicrobials has been a conventional therapy in treatment of uterine infections in cattle although the use of antibiotics has not been without controversy. Debate continues regarding antimicrobial efficacy, effects on future fertility, risk for bacterial resistance and residues. The proper use of antimicrobials to treat uterine infections must first begin with an appropriate diagnosis and thorough understanding of the immunology of the uterus, the pathophysiology of uterine infections, and the properties of the various antimicrobial agents that may be used therapeutically.

Normal uterine involution

Understanding normal uterine involution is vital to understanding and defining postpartum disease in cattle. Lochia are normally present until 14 to 23 days postpartum.[1] After placental detachment, uterine involution is complete in an average of 39 days in normal cows. By day 6 postpartum, caruncle septa are disorganized, and by day 15 caruncles are completely sloughed due to necrosis. By day 26 to 30 postpartum, the surface of the endometrium is covered by new endometrium.[2] Cervical involution is slower than uterine involution and by day 15 postpartum, the diameter of the cervix normally exceeds that of the uterine horns. Reported times for gross involution of the uterus and cervix vary from 25 to 47 days. Complete histologic uterine involution takes longer than palpable involution and occurs at 42 to 50 days.[1]

Infection of the bovine uterus

The majority of cattle experience bacterial contamination of the uterus at the time of parturition. In the normal cow, the uterus is cleared of this bacterial contamination by four weeks postpartum and when these bacteria are not cleared by the cow's defense mechanisms, a uterine infection ensues. Numerous bacteria have been isolated from the cow's postpartum uterus, some of which may be incidental and not cause problems. Uterine infections are most commonly due to *Arcanobacterium pyogenes*. The gram negative anaerobes *Fusobacterium necrophorum* and *Bacteroides melaninogenicus* are frequently associated with *A. pyogenes*. Other organisms that may be associated with uterine disease in the cow include *Pseudomonas aeruginosa*, staphylococci, hemolytic streptococci, coliforms, etc. *Clostridium sp.* may occasionally infect the uterus and cause a severe gangrenous metritis or tetanus. Uterine infections in the cow are associated with retained fetal membranes, dystocia, and delivery of twins.[3]

Metritis is the result of severe inflammation involving all layers of the uterus – endometrial mucosa and submucosa, muscularis, and serosa. Metritis usually develops during the first week after calving and is associated with dystocia, retained fetal membranes, and calving trauma. These cattle may be septic and present with fever, depression, and anorexia. A copious amount of fetid vaginal discharge may also be present. Endometritis is characterized by inflammation of the endometrium extending no deeper than the stratum spongiosum. Cows with endometritis are usually not systemically ill, and bacteria are usually eliminated after a few estrous cycles. Endometritis is characterized by a mucopurulent or purulent uterine discharge associated with a chronic uterine infection, usually later than 3 weeks postpartum.[1]

Diagnosing uterine infections

Uterine infections are most commonly diagnosed at routine examination of postpartum cows, at breeding, or upon examination of a sick cow. A diagnosis of metritis or endometritis may be made by a variety of methods which include: clinical signs, rectal palpation, vaginoscopy, ultrasonography, uterine culture, uterine cytology, and uterine biopsy. Clinical signs of uterine infection vary with the virulence of the causative organism and the severity of predisposing conditions of the cow. Uterine discharge may vary considerably in color; however, discharges are not usually considered abnormal unless the uterine fluid is fetid or if the cow has clinical signs of sepsis. Palpation per rectum is the most commonly used technique for evaluating the degree of uterine involution prior to breeding and is used to diagnose endometritis through evaluation of the size and consistency of the uterus and cervix and the presence of fluid within the lumen of the uterus. If involution is normal, fluid should not be palpable within the uterine lumen by 14 to 18 days postpartum. In cases of severe metritis, the uterus will be enlarged and friable with occasional adhesions between the uterus and other organs or the body wall. Although rectal palpation is commonly

used for diagnosis of endometritis, it is neither sensitive nor specific. The evaluation of purulent exudate with the aid of a vaginal speculum may also be a useful tool for diagnosing endometritis. Ultrasonography has also been used to evaluate and characterize intralumenal fluid within the uterus as well as the thickness of the uterine wall. Uterine infections are associated with intrauterine fluid with echogenic particles and a variably thickened uterine wall. Bacterial culture is rarely used as treatment must be initiated before culture results are available. Culture is more commonly used in cases where cows fail to respond to treatment. Endometrial biopsy is not used as commonly in cattle as it is in mares and is reported to have a detrimental effect on future fertility.[3,4]

Acute-phase proteins have been evaluated as markers for endometritis in the postpartum cow. These acute-phase proteins include peripheral blood haptoglobin and α_1-acid glycoprotein. Haptoglobin is synthesized in the liver in response to tissue damage and binds free hemoglobin to protect from the oxidative activity of hemoglobin. Serum haptoglobin concentrations increase in dairy cows with acute metritis, but do not increase significantly in cases of chronic metritis.[5,6] Another study showed that cows with acute postpartum metritis generally have low concentrations of plasma haptoglobin while cows with severe metritis had consistently higher levels.[7] A more recent study indicated that cows with $\geq$1 g/L of haptoglobin on day 3 postpartum are 6.7 times more likely to develop severe or mild metritis. This study also suggests that an acute phase inflammatory response precedes clinical metritis and that haptoglobin screening may assist in the early detection of metritis.[8] The α_1-acid glycoprotein has been evaluated as well, but the results were less diagnostic than those for haptoglobin.[7] The significance of these acute phase proteins in the diagnosis of endometritis or metritis is not fully understood. Intrauterine oxygen reductase potential (Eh) and pH have also been explored as a means to assess the level of bacterial contamination within the lumen of the uterus. The Eh values fell in the presence of infection which created an anaerobic environment within the uterus. It is thought that the drop in Eh is due to either bacterial metabolism or increased oxygen consumption by polymorphonuclear (PMN) cells.[9] The pH of uterine discharge collected from cases of endometritis varied from 6.9 to 7.3 which favors the growth of *A. pyogenes*.[10,11]

Immunology of the bovine uterus

The uterine defense mechanisms against contaminating bacteria are maintained in many ways which include 1) anatomically by the simple or pseudostratified columnar epithelium covering the endometrium, 2) chemically by the mucoid secretions from the endometrial glands, and 3) immunologically by PMNs and humoral antibodies.[10] Disruption of these natural defense mechanisms allows for invasion and colonization of the endometrium by opportunistic pathogens. Inflammation of the bovine endometrium can occur following coitus, artificial insemination, or more commonly postpartum.

Uterine cellular immunity

In the uterus, the cellular defense against bacterial invaders is provided by uterine leukocytes. The PMN population within the uterine lumen increased after experimentally-induced uterine infections.[12,13] At approximately 48 hours postpartum in unassisted calvings, leukocytes begin to accumulate in the uterine lumen along with bacterial contaminants.[14] This is the beginning of the normal process of uterine involution. In cases of metritis there is an initial decrease in the phagocytic activity of uterine PMNs.[10,15] Two to three weeks later when clinical recovery has occurred, the phagocytic activity increases which also coincides with lower numbers of bacteria in the uterine lumen.[10,16]

The cellular immune response of the uterus may be negatively affected by some treatments commonly used to treatment postpartum disorders in the cows.[10,17] It has been found that manual removal of fetal membranes, intrauterine antiseptics and disinfectants, and intrauterine antibiotics may inhibit or suppress uterine leukocyte phagocytic activity for several days.[10,18] It has also been noted that Lugol's iodine and polyvinyl-pyrrolidone both cause necrosis of the endometrial epithelium and stimulate uterine defense mechanisms and release of prostaglandin $F_{2\alpha}$.[19-22] Although these agents stimulate uterine defense mechanisms, they also cause endometrial fibrosis and thus should not be used as an intrauterine therapy.[10,19,23]

Elevated blood progesterone concentrations have been found to inhibit both uterine and peripheral blood neutrophil phagocytic activities. The numbers of peripheral blood neutrophils increase slowly from about six weeks prior to parturition and reach a peak on the day of calving.[16,24,25] However, maternal and fetal cortisol at the time of calving may suppress neutrophil function.[26-28] Immediately postpartum, the phagocytic activity of blood neutrophils declines within the uterine lumen.[10,29] The cellular defense mechanisms are preserved by an increase in the number of PMN cells.[10,24] During the first three weeks postpartum, the number of peripheral blood PMNs declines and is likely due to the migration of these cells into the mammary gland and uterine lumen.[24,30,31] In addition, the phagocytic activity of the PMNs declines which is more marked in older versus younger cows.[10,30,31] There is some disagreement as to whether there is a decrease in the phagocytic activity of neutrophils in the uterine lumen versus the peripheral blood.[32-34] Other leukocytes are present in the endometrium of all animals. Lymphocytes are found

within the endometrial epithelium in both cycling and non-cycling ewes and heifers with little variation in numbers at different stages of the estrous cycle.[35,36]

Uterine humoral immunity

Protective immunoglobulins have been found in the bovine uterine secretions.[37-41] Immunoglobulin A (IgA) is produced locally from the mucosa of the bovine uterus whereas IgG is produced from two sites. A portion of IgG_1 is produced locally in the endometrium while the remaining IgG_1 and all of IgG_2 is obtained from peripheral circulation.[10,37,42] Experimental uterine infections with pathogenic bacteria have demonstrated immunoglobulins in cervical and vaginal secretions that appear in the order IgM, IgA, and IgG and disappear in the order IgM, IgG, and IgA.[10,43] The concentrations of each immunoglobulin depends upon the site of sampling with IgG predominately found in uterine lumen and IgA in the vagina.[10,13,43,44] Both IgG and IgM concentrations in lochia from healthy cows fall after calving.[45] In cows with postpartum disease, both IgA and IgG concentrations in uterine fluids increase quite rapidly as endometritis develops. However, IgM remains low in cattle with endometritis.[46]

Intrauterine therapy

A variety of antibiotics and antiseptics have been infused into the uterus of cows to treat postpartum infections. Intrauterine antimicrobials are used in order to achieve high concentrations at the site of infection but are usually unable to penetrate any deeper than the endometrium.[1] The intrauterine use of antimicrobial agents is controversial as some have found intrauterine treatment to be beneficial while others have found these agents to have no effect or a detrimental effect. The bovine uterus is an anaerobic environment. Thus, antibiotics that are chosen for intrauterine infusion must be active in the absence of oxygen. Additionally, most antibiotics depress the activity of uterine neutrophils and interfere with uterine defense mechanisms.[3] Thus, one must carefully evaluate the evidence regarding intrauterine antimicrobial use and carefully consider both the advantages and disadvantages associated with therapy.

Historically, intrauterine use of antimicrobials has been a common therapy for treatment of uterine infections. Antimicrobials reportedly used for uterine infections include tetracycline, penicillin, cephapirin, chloramphenicol, Lugol's iodine, gentamycin, spectinomycin, sulfonamides, nitrofurasone, povidone iodine solution, urea, and chlorhexidine.[1] Most of these compounds are not approved for intrauterine use and have no published withdrawal times. There are also reports that intrauterine infusion of antibiotics cause drug residues in milk.[47,48] Additionally, regulatory guidelines must be adhered to for extralabel use of antimicrobials in food animals. Intrauterine therapy is considered an extralabel use, and thus may be prohibited for many antibiotics, particularly in the United States.

The organisms that cause most postpartum infections are usually sensitive to penicillin. However, bacterial contaminants present within the uterus during the first several weeks postpartum produce penicillinase which makes penicillin useless if used locally in the early (less than 30 days) postpartum period. By 30 days postpartum, the contaminating bacteria are usually eliminated and intrauterine treatment with penicillin is more likely to be effective.[3] Other factors may also affect the efficacy of intrauterine antibiotic therapy. Uterine lochia present during uterine infections contain organic fluids and debris that can render certain antibiotics, such as sulfonamides, ineffective.

More recently, oxytetracycline has been the antimicrobial commonly used for intrauterine therapy.[3] However, one study indicated that most isolates of *A. pyogenes* are resistant to oxytetracycline. This study also showed that large doses of intrauterine oxytetracycline did not affect the frequency of isolation of *A. pyogenes*.[3,49] In addition, oxytetracycline and Lugol's iodine are quite irritating and are reported to cause coagulation necrosis of the endometrium.[3,50] Although some studies indicate improved reproductive performance with the use of intrauterine oxytetracycline, it has been speculated that this improvement may be due to local prostaglandin production due to chemical irritation of the endometrium.[51]

In general, intrauterine infusion of antimicrobials has failed to show any increase in reproductive performance.[1] Two large field studies evaluated the use of cephapirin benzoate in cows with clinical endometritis and demonstrated an improvement in reproductive performance.[52-54] But other studies have shown no improvement in reproductive performance when evaluating intrauterine administration of cephapirin benzoate.[53] The appropriate use of intrauterine antibiotics to treat uterine infections still remains controversial as only a limited number of studies indicate the efficacious use of intrauterine antibiotics.

Numerous antiseptics have been used to lavage the postpartum bovine uterus with iodine and chlorhexidine solutions being most commonly used. Many of these solutions are quite irritating to the endometrium and are thought to stimulate endogenous prostaglandin release. One study showed that the incidence of retained fetal membranes and endometritis was reduced in cows that received 500 mL of 2% Lugol's iodine immediately after calving and again 6 hours later. However, this study did not evaluate the future reproductive performance of these treated cows.[3,55] Another study evaluated the use of 50 to 100 mL of 2% povidone iodine solution in the uterus one

month postpartum and found that the reproductive performance of normal cows was not improved and that the treatment was detrimental to the fertility of cows with endometritis.[3,23]

Systemic antibiotic therapy

Cattle with metritis often suffer moderate to severe illness. These cattle are often septic with fever, depression, and anorexia. A variety of antibiotics have been recommended for parenteral use in cattle suffering from uterine infections. Penicillin or one of the synthetic penicillin analogs and ceftiofur are among the more common antibiotics used systemically in cattle suffering from metritis. Systemic use of oxytetracyline may not be efficacious because of the difficulty in achieving the minimal inhibitory concentration (MIC) required for *A. pyogenes* in the uterine lumen.[3] However, one study found clinical improvement of cattle suffering from metritis with the use of tetracycline at 10mg/kg.[56]

Ceftiofur is a third-generation cephalosporin that has broad-spectrum activity against gram negative and gram positive bacteria.[57] Ceftiofur is reported to reach all layers of the uterus without causing violative residues in milk. Ceftiofur is approved in the United States for systemic administration to lactating cows affected with metritis.[3] A subcutaneous dose of ceftiofur at 1mg/kg in postpartum cows results in a concentration of ceftiofur and its active metabolites in plasma, uterine tissues, and lochia higher than the MIC for most of the common pathogens involved in metritis.[58] One study demonstrated that ceftiofur administered at 2.2mg/kg daily for five days was effective in treating cows with metritis.[57] Another study supported these findings and showed ceftiofur administered at 2.2 mg/kg once daily for five days is as effective for treating metritis as procaine penicillin G or procaine penicillin G with intrauterine infusion of oxytetracycline.[59]

Because of the reported lack of efficacy and potential detrimental effects of future fertility, intrauterine infusion of antibiotics is not a favored treatment for most cases of metritis. Certain systemic antibiotics have demonstrated their effectiveness at treating uterine infections in cattle. Thus, most cases of metritis, especially cows that are toxic, should be treated with systemic antibiotics such as penicillin or ceftiofur.

Conclusion

There are no antibiotics currently approved for intrauterine administration in the United States. Intrauterine infusion of antibiotics leads to contamination in milk and tissues for which appropriate withdrawal times have not been ascertained. In addition, the assays used on farms to detect antibiotics in milk may not be accurate. Although some studies indicate a positive response to therapy with the use of intrauterine antimicrobials, most studies do not show an improvement in reproductive performance or clinical signs of disease when comparing intrauterine antimicrobial therapy and systemic antibiotic therapy. This information, in conjunction with concerns regarding uterine or endometrial damage and withdrawal times following the use intrauterine antimicrobials, suggests systemic antibiotic therapy as the best treatment for many cases of cows with uterine infections.

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Cystic ovarian disease in dairy cattle

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Abstract

Practitioners involved in the delivery of veterinary care and who provide consultation to dairy cattle owners must be able to effectively diagnose and treat common conditions which impact the economic stability of the dairy enterprise. This manuscript provides the practitioner with the information to understand the pathophysiology of cystic ovarian disease, choose an appropriate treatment and suggest possible management changes to minimize the economic impact of this disorder.

Keywords: Cystic ovarian disease; anestrus; infertility; GnRH; cattle

Introduction

Cystic ovarian disease (COD) has been recognized as a frequent cause of subfertility and poor reproductive efficiency in cattle for almost 100 years and is still considered to be one of the most economically important reproductive conditions affecting dairy cattle worldwide. The major causes of economic loss are due to increased days open in the postpartum period which extends the calving interval, costs associated with treatment and higher culling rates in affected animals.[1,2] Cystic ovarian disease has also been shown to decrease the pregnancy rate to subsequent AI which leads to an increase in services per conception resulting in increased semen costs.[3]

Terms which have been used to describe the condition of persistent anovulation of preovulatory follicles include: cystic ovarian disease, cystic ovarian follicles, cystic ovarian degeneration and cystic ovaries. The most common term used in the literature to describe this condition is cystic ovarian disease. As we understand more about this condition and management practices have changed we should revisit the use of the term cystic ovarian disease. The most recent term used to describe this ovarian dysfunction is cystic ovarian follicles (COF).[1,3] Since "cysts" are often diagnosed in the absence of any obvious clinical signs the term "disease" should likely be replaced by "follicles" as it more accurately describes the condition.[3]

Definition

The classical definition of COD in cattle is the presence of an anovulatory structure on the ovary which is > 2.5cm in diameter and has persisted for at least 10 days in the absence of a corpus luteum (CL).[4] As more knowledge is gained regarding COD the previous definition requires refinement. The size limit of 2.5cm is arbitrary and would exclude cystic follicles which are smaller than 2.5cm. The dominant follicle of dairy cattle typically ovulates on average at a size of 1.6-1.9cm.[3] The classic definition that requires presence for 10 days should also be questioned due to the fact that cystic ovarian follicles have been shown to be dynamic structures which change over the course of time. Also cows diagnosed with COD are generally not palpated again in 10 days to totally fulfill the classical definition. The necessity for the absence of a CL is also not universally fulfilled. Cysts which are non-steroidogenic and thus hormonally inactive may not influence the estrous cycle and thus could be found in the presence of a CL. Most cows today however are diagnosed with COD on the basis of a single rectal palpation or ultrasound examination and no attempt is made to assure the structure has been present for 10 days in the absence of a CL.

A recent term and definition put forth in the literature to more accurately describe this condition is cystic ovarian follicles. Cystic ovarian follicles are defined as follicles with a diameter of at least 2 cm that are present on one or both ovaries in the absence of any active luteal tissue and that clearly interferes with normal ovarian cyclicity.[3] This definition more clearly defines the condition in relation to our current understanding and its impact on reproduction.

Cysts are further classified as being follicular cysts or luteal cysts depending on the degree of lutenization and the level of progesterone secretion. Both are considered to be different forms of the same condition with luteal cysts being a follicular cyst which has undergone some lutenization.[2] Follicular cysts do not secrete progesterone whereas luteal cysts secrete varying amounts of progesterone however an absolute threshold has not been determined.[3] The ability to accurately classify each cyst is subject to personal interpretation based on clinical as well as laboratory findings.

Ultrasound can be a very useful tool with which to gather information regarding the classification of cysts. Follicular cysts typically have a thin wall ($\leq$ 3 mm) whereas luteal cysts typically have a thicker wall ($\geq$ 3 mm). The follicular fluid is often hypoechoic in follicular cysts whereas in luteal cysts it may contain echogenic strands creating a web-like appearance.[5]

Incidence

The incidence of COD in dairy cattle varies amongst several studies but is typically between 5-19% with mean of 10-12%.[2] The incidence of COD could likely be even higher based on the findings that as many as 60% of cows that develop COD recover spontaneously prior to their first postpartum ovulation and could easily remain undiagnosed. The majority of COD is diagnosed by routine rectal palpations during the first 60 days postpartum at which time cows are being examined prior to breeding. Cases are also commonly diagnosed between 120-210 days postpartum. These cases are typically diagnosed in cows which have been presented for examination after extended periods of anestrus.

There is a genetic predisposition for COD in dairy cattle, however the heritability is low at 0.07 to 0.12.[6,7] Cystic ovarian disease seems to occur more often in certain cow families. Genetic selection attempting to remove sires who produced daughters that developed COD from the breeding pool has been shown to significantly reduce the incidence of COD in Swedish herds.[3] Reduction in the incidence from 10% to 3% was achieved by selection against sires that produced daughters with COD.[8] Genetic selection as a prevention for COD will be a lengthy endeavor due to the low heritability but can be effective.

With routine use of synchronization programs (i.e., Pre-sync, Ovsync) during the voluntary waiting period to synchronize the first postpartum AI the incidence of COD as diagnosed by rectal palpation could likely be lower than previous studies have identified. However, as the modern dairy cow is under tremendous dietary and production stressors which can predispose her to COD the apparent affect of these programs on clinical incidence may be modulated.

Clinical signs

Behavioral signs seen in cows with COD are variable but can generally be classified into two groups, anestrus and nymphomania. The most common clinical sign observed in cows with COD is anestrus, this is especially evident in the early postpartum period. Approximately 80% of cows that develop COD early in the postpartum period exhibited anestrus.[4] These cows are often presented for examination after failure of the herdsman to detect normal postpartum cycling activity. Nymphomania is yet another clinical sign which can be seen in cows with COD. These cows often attempt to ride other cows but generally will not stand for mating themselves.[4] Approximately 10% of cows affected with COD show signs of nymphomania. It appears that as the number of days following calving at which COD is diagnosed increases the likelihood of nymphomania being a clinical observation also increases.[4] Irregular estrous cycles can also frequently occur in cows with COD which often leads to inappropriate breeding of these cows based on poor or weak signs of estrus.

Pathogenesis

A dysfunction in the normal hypothalamic-pituitary-gonadal axis leading to ovulation failure is the most common accepted mechanism of COD.[1,2,9-11] The precise mechanisms leading to the aforementioned dysfunction have yet to be fully elucidated. It is believed that there is a multi-factorial cause with genetic, phenotypic, environmental and management factors involved.[3]

The most widely accepted hypothesis involves the altered release of luteinizing hormone (LH) from the pituitary gland. The pre-ovulatory surge of LH is either absent, insufficient in magnitude or is improperly timed whereas the dominant follicle does not ovulate leading to cyst formation.[1-3] There does not appear to be a reduction in GnRH content in the hypothalamus or a reduction in GnRH receptors in the pituitary.[3] Luteinizing hormone content in the pituitary also does not appear to be reduced in cows with COD.[3,12] Normally pre-ovulatory follicles secrete estrogen which has a positive feedback on the hypothalamic-pituitary axis causing release of LH which is responsible for the subsequent ovulation. There appears to be a lack of responsiveness of the hypothalamus to the positive feedback mechanism of estrogen leading to the altered release of GnRH and/or subsequently LH causing the anovulatory state of COD.

Predisposing factors

Numerous factors have been associated with the development of COD in cows. This condition appears to more commonly affect high producing dairy cows in their second through fifth lactation. Early in lactation when the cow is often in a negative energy balance metabolic disturbances are more common and are often followed by COD. There appears to be a higher incidence of COD during winter months, however photoperiod does not appear to have an effect on the hypothalamic-pituitary-ovarian (HPO) axis. Other factors which have been associated with depression of GnRH/LH release and subsequent cyst formation include uterine infections, retained fetal membranes, lameness and stress.[3,11] Postpartum uterine infections are thought to stimulate cortisol secretion which can suppress the pre-ovulatory surge of LH leading to anovulation and subsequent cyst formation.[1] The associated endotoxins and inflammatory mediators can disrupt the normal hormonal pathways that ultimately control ovarian function including ovulation.[13] The role of stress in COD is believed to be related to the release of cortisol which appears to block the estrogen induced LH surge.[1,10,14]

Diagnosis

The diagnosis of COD has historically been made based on the finding during rectal examination of the cow along with her reproductive history. However the collective findings of a rectal examination, an ultrasonographic evaluation of the reproductive tract including the ovaries, progesterone concentrations in blood or milk and behavioral abnormalities will allow for a more accurate diagnosis. The accuracy with which a skilled palpator can identify the type of cysts based on palpation alone is relatively poor.[15] The dynamic nature of both cysts and developing corpora lutea can complicate the diagnosis when palpation alone is used. A study by Farin et al., showed 10% of cows that were diagnosed as having cysts based on rectal examination were found to have a structure consistent with a normal corpus luteum by transrectal ultrasound examination.[16] In one study evaluating the use of ultrasound the accuracy of a correct diagnosis being made was 74% of follicular cysts and almost 90% of luteal cysts.[17] Progesterone concentrations have been shown to correlate very well with cyst wall thickness with 3 mm being the threshold between follicular and luteal cysts.[1,18] When one combines progesterone concentrations in addition to rectal examination and ultrasound findings the accuracy of diagnosis of the cyst type approaches 100% however this is rarely done outside of research settings. Although using progesterone testing to accurately determine the cyst type would aid in treatment decisions it is rarely used in practice situations due to the economic considerations.

Treatment options

Probably the oldest treatment of COD in cattle is manual rupture of the cyst via rectal palpation. With the advances in our understanding of COD and the availability of effective medical options this treatment can no longer be recommended. The possibility of oviductal or ovarian bursal adhesions arising secondary to the trauma associated with manual rupture and their affects on subsequent fertility are too great to ignore.[4]

Hormone therapy aimed at either causing (GnRH) or mimicking (human chorionic gonadotropin; hCG) an LH surge can be used to treat follicular cysts. Of these two, GnRH is generally chosen first due to its small molecular size which reduces the likelihood of an immune reaction.[1,10] After an injection of GnRH a surge of LH from the pituitary occurs within 2 hours.[19] This LH surge can cause lutenization of follicular cysts which will undergo spontaneous luteolysis in about 18 days at which time a normal estrous cycle begins. Another possibility following GnRH treatment in cows with follicular cysts is ovulation of a dominant follicle followed by a subsequent normal luteal phase. Since cystic cows continue to have follicular waves, the response to GnRH is likely due to ovulation of a dominant follicle present with recruitment of a new cohort of follicles rather than lutenization or regression of the cysts.[20] The subsequent increase in progesterone concentrations causes the re-setting of the normal HPO axis and resumption of normal cyclicity in most cows. In one study that evaluated the effectiveness of a single injection of GnRH for treatment of cows with ovarian cysts, 72% of cows resumed normal cycling within 20 days of treatment compared to 16% of control cows.[19] However other studies have not borne out the same results. A study that evaluated the effectiveness of GnRH as a sole treatment for follicular cysts showed no difference in treated animals versus controls. The lack of agreement between numerous studies evaluating the efficacy of GnRH is likely due to the lack of control animals and the number of cows which recover spontaneously. There was no difference in the period of time between treatment with GnRH and resolution of the cyst or in the period of time until a CL was evident.[21] This study brings to light the high incidence of spontaneous recovery and somewhat brings into question the effectiveness of GnRH alone as a sole treatment for cows with COD. Timing of treatment in the postpartum period does not appear to affect treatment response. In one study, cystic cows were treated with GnRH either before or after 60 days following calving. There was no difference in treatment response in the two groups however there were no control animals with which to compare.[22] In accurately diagnosed cases a relatively large percentage of cows return to cyclicity following an injection of GnRH, however some of this response could be attributed to spontaneous resolution.

A recent pharmacokinetic study attempted to find the optimal dose of GnRH for treatment of cows with COD. The dose of GnRH which was found to guarantee production of a critical maximum plasma LH concentration of 5 ng/ml was 74 ug of GnRH.[23] Therefore the standard 100 ug dose of GnRH used to treat cows with COD was found to generate a LH concentration of 5.86 ng/ml and should be adequate in most cases of COD. [23]

Human chorionic gonadotropin has been used successfully to treat refractory follicular cysts that fail to respond to GnRH. It has LH-like properties and causes the cyst to lutenize and begin producing progesterone. Once the cyst has lutenized it can then be treated with prostaglandin to restore the normal cyclical pattern. Its use is often relegated to cases in which GnRH has failed to render a cure. Its use has occasionally been noted to stimulate an immune reaction however the importance of this reaction is poorly understood.[1]

Prostaglandin is the treatment of choice for luteal cysts and cysts that have undergone lutenization after being treated with GnRH or hCG.[1,2,10,20] Prostaglandin has no effect on follicular cysts so it is important to accurately diagnose the type of cyst before using prostaglandin alone. After prostaglandin administration luteal

cysts regress with estrus occurring in 90% of cows by day 8 post treatment. Prostglandin is commonly used in the treatment of cysts after a previous injection of GnRH as part of an Ovsync protocol.

Protocols involving a series of hormonal injections aimed at treating the cysts and restoring the cow to normal cyclicity have been proposed.[1,11,24-27] The classical Ovsync protocol has been employed as a treatment for cysts irrespective of their type. The rational in using an Ovsync protocol is to both treat the cyst and eliminate estrus detection and breed the cows with timed AI.[11,24-27] Progesterone levels in cows with COD which are treated with GnRH are elevated five days after treatment and therefore could be treated with traditional Ovsync with good results.[24] When cows with COD are subjected to the Ovsync protocol pregnancy rates to subsequent timed insemination has range from 17-25%.[25,28]

Use of progesterone as a treatment for cows with COD has been proposed for over 40 years.[29] Now that progesterone impregnated vaginal pessaries have been approved for use in lactating dairy cattle in the United States recent emphasis has been placed on their use in the treatment of COD. Progesterone administration has been shown to re-establish the normal feedback mechanisms involving the HPO axis and allow cows with COD to resume normal cyclicity. The duration of progesterone treatment which is sufficient to re-establish normal hypothalamic responsiveness to estradiol appears to be as short as three days.[30] There was no difference in the pregnancy rates of dairy cattle with COD when treated with either the Ovsync protocol or use of a progesterone-releasing pessary for seven days with prostaglandin administration at the time of pessary removal followed by breeding after heat detection suggesting that use of intravaginal progesterone as a treatment for COD can be effective.[27] Use of progesterone-releasing pessaries in combination with the Ovsync protocol has been studied as a treatment for cysts as well. Results showed an increase in pregnancy rates in cystic cows treated with Ovsync plus progesterone (37.5% pregnancy rate) compared to Ovsync alone (16.7% pregnancy rate).[26]

In a recent study, the effectiveness of the opioid antagonist naloxone as a treatment for cows with COD was examined.[31] It has been shown that stress may be a contributor to the pathogenesis of COD in cattle. Endogenous opioid peptides are involved in many responses to stress including the regulation of various endocrine systems.[31] Endogenous opioid peptides are believed to block the release of GnRH from the hypothalamus as well as the estrogen-induced LH surge.[14] It has been shown that administration of the opioid antagonist naloxone results in elevated LH release in cattle under various physiological states.[32] Cows diagnosed with COD were treated with naloxone as well as the GnRH agonist buserelin. In this study 77.5% of treated cows had begun cystic regression as viewed with ultrasonography or had begun cycling normally within two weeks post-treatment.[31] Further investigation of the use of naloxone is warranted and specifically its use in the absence of GnRH products which confound the interpretation of this particular study.

Conclusion

Cystic ovarian disease remains an important postpartum condition affecting dairy cattle with a substantial economic impact on the modern dairy farm. Although much effort and research has been placed on elucidating the precise mechanism(s) leading to COD its exact cause remains unclear. Currently the most effective treatments for COD appear to be those which are capable of resetting the HPO axis thereby re-establishing normal feedback mechanisms which results in normal cyclical patterns. Both the Ovsync protocol, the use of intravaginal progesterone, or the combination of the two appear to be the most practical and effective treatments for most modern dairy operations. Due to the fact of a high incidence of spontaneous recovery in cows with COD it has made it difficult to interpret apparent response to various therapies. Further research into the cellular and molecular events that are occurring in cows with COD and the interactions of various stressors will hopefully provide us more answers to this very common condition.

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Legal issues in the pharmaceutical management of bovine reproduction

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Abstract

The ability for the bovine practitioner to implement reproductive management programs is scientifically well-based and clinically proven. However, as is being encountered in many areas of food animal medicine, science is no longer the major determinant of how a veterinarian practices or how a producer manages. Extra-label drug use (ELDU) allowed under the Animal Medicinal Drug Use Clarification Act was not extended to drugs used for production purposes, such as reproduction. Therefore, ELDU of drugs for reproductive purposes is not technically allowed in the Code of Federal Regulations (CFR). Compounding of drugs from raw pharmaceutical agents, except for a few specifically listed antidotes, is a violation of the CFR. Further, the use of illegally compounded products in food animals is ill-advised not only due to the legal and liability issues, but also because of a potential consumer perception issue. A final point to consider is the consumer reaction when current bovine reproductive management programs are presented to the consuming public by anti-agriculture activists.

Keywords: AMDUCA, compounding, management, reproduction, extra-label

AMDUCA and ELDU in reproductive management protocols

The Animal Medicinal Drug Use Clarification Act[1] (AMDUCA) of 1994 and the implementing regulations[2] were limited to drugs used for therapeutic purposes only. Numerous comments to the proposed rule supporting the extension of the regulations to drugs used for reproductive management were considered but denied in the final rule. It is important to note that AMDUCA applies only to Food and Drug Administration (FDA or the Agency) approved drugs. The current situation has resulted in any extra-label use of reproductive drugs being illegal. The U.S. FDA has adopted an unofficial position of regulatory discretion. Any official position would require the generation and publication of a Compliance Policy Guideline (CPG) and the FDA has not indicated that it would consider taking this action.

The FDA's reasoning behind placing low regulatory priority on the issue of using drugs approved for reproductive purposes in a manner other than on the label is that these drugs have been proven safe and effective, and the dose, route and species are not altered. For example, the drug most often used in an extra-label manner is gonadotropin releasing hormone (GnRH). GnRH is a decapeptide and poses virtually no risk of residues nor any target animal safety issues. For these reasons, the Agency has suggested that they currently will not pursue any actions directed at extra-label use of reproductive hormones. At the same time, the FDA has indicated that they would be glad to entertain and possibly facilitate the approval of additional label claims for GnRH products so that the need for ELDU would be minimized or eliminated.

One final point which should be made regarding ELDU of reproductive drugs is that of client informed consent. ELDU has been the basis for at least one major lawsuit against a veterinary practice. In today's litigious society, ensuring that clients are aware of the extra-label nature of any use is appropriate. Having an informed and consenting client is ever more important when such ELDU is not in compliance with the Code of Federal Regulations.

Compounded drugs

Compounding medications from unapproved drugs or raw pharmaceutical agents is illegal. References to this can be found in the AMDUCA regulations[1] and in an FDA CPG.[3] There are exceptions in which drugs can be compounded under this CPG, but these are limited to certain specified antidotes only. The most common drug compounded for use in reproductive management protocols is some form of estrogen. Estrogens have been proven effective in initiating follicular waves both in embryo transfer programs and in routine breeding management systems.

However, in this case science has surpassed the veterinarian's armamentarium of approved drugs and, while the use of compounded estrogens may be tempting due to effectiveness, the potential for regulatory action and consumer backlash makes the use of compounded estrogens ill-advised. Currently the FDA lists one estrogen (diethylstilbestrol) on the list of drugs prohibited for extra-label use in food animals. An estrogen (estradiol cypionate) which was registered in 1953 but never approved has been withdrawn from the market by the sponsor based upon an impending request for an official approval application.

Of all the dugs used in cattle, both label and extra-label, the single drug with the greatest "headline potential" is any form of estrogen. The average consumer's lack of familiarity with animal husbandry and modern agricultural production practices, make the consideration of consumer acceptance relevant to the discussion of both

ELDU and the use of compounded drugs in food producing animals. This significant downside to the use of a drug compounded from unapproved products (specific antidotes are exceptions) makes such drug use illogical and irresponsible.

Consumer/public perception

Animal agriculture has numerous detractors in the form of activists. The issues which are used to malign the animal agriculture industries to the consuming public include, but are not limited to, animal welfare and factory farms. Because less than 1% of the population of the U.S. is involved in agriculture, the activist groups find the consuming public easily misled about commonly accepted, well proven, welfare-friendly and accepted management practices. The portrayal of modern agriculture as factory farming or intensive farm animal production is negatively compared to the typical small farming operation of 50 and 60 years ago. With this as background, some of the current reproduction management protocols which utilize multiple injections of various products coupled with timed artificial insemination would represent easy targets for malicious mis-representation to the uninformed public. The point of this thread is not to argue that the management protocols are indefensible, rather that the discussion will have to ultimately be directed at a consuming public unfamiliar with any aspect of animal agriculture. The science-based discussions held with colleagues regarding choice or efficacy of protocol will not apply when the production practices are discussed in a public forum. The impacts that these programs have on animal productivity, operational sustainability and the production of a safe, abundant and affordable food supply will be the key communication points.

Summary

Extra-label use of drugs in reproductive management programs is not allowed under AMDUCA. Therefore, ELDU of drugs for reproductive purposes is not technically allowed in the CFR. Compounding of drugs from raw pharmaceutical agents, except for a few specifically listed antidotes, is a violation of the CFR. In addition, the use of illegally compounded products in food animals is ill-advised not only to the legal and liability issues, but also because of a potential consumer perception issue. A final point to consider is the consumer reaction when current bovine reproductive management programs are presented to the consuming public by anti-agriculture activists.

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Applying ultrasound to the individual dairy cow and herd level reproductive management

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Modern dairy cattle have been described as subfertile.[1] Implementing timed breeding synchronization programs (TAI) can improve heat detection efficiency, but conception rates can remain low. The majority of synchronization programs are initiated regardless of where the cow is within her estrous cycle, and it has been shown that conception rates vary greatly depending upon when these programs are started in relation to the cow's estrous cycle.[2] Most important in predicting the stage of a cow's estrous cycle is identifying the presence of an active corpus luteum (CL). Ultrasound has been found to be far superior to rectal palpation in predicting an active CL and high progesterone levels,[3] and thus better predicting when cows fall into the optimum period to initiate or continue synchronization programs. By using ultrasound, cows can be assessed and synchronization programs can be modified when cows fail to respond to the first GnRH injection.

Ultrasound can also be used as a heat detection aid, especially for dairies that utilize tail chalk. Accuracy of heat detection when using tail chalk can vary, and often cows in diestrus or even pregnant are bred. Using ultrasound, cows with an active CL can be avoided, and only cows appearing in estrus will be bred.

Incorporating ultrasound intensively into TAI and estrus detection programs can improve pregnancy rates dramatically. Individual cow management along with techniques and strategies to apply these protocols on any size dairy will be discussed.

Keywords: Ultrasound, synchronization, timed AI

Introduction

Modern dairy cattle have been described as subfertile[1] and the expanding dairies of the western U.S. seem to be experiencing some of the greatest reproductive inefficiency. According to DHI-Provo data, since 1990, days open have increased by more than 30 days for dairies in the western United States, with conception rates declining almost 25% during that same period. Heat detection rates followed the same downturn during the 90's, but with greater implementation of TAI, conception rates have since risen to values approaching 45 to 50%. While the greater use of synchronization programs has improved heat detection efficiency, conception rates have still continued to decline. It is not uncommon to find herds in the western United States with conception rates to timed breeding programs mired in the 20% range. There are multiple contributing factors to this problem, such as those related to abnormal reproductive physiology, poor timing of initiation, and non-compliance to synchronization protocols.

Materials and methods

In order to achieve acceptable reproductive performance additional management tools need to be implemented to overcome this subfertility. Timed breeding synchronization programs are one such tool but conception rates with these programs, especially in expanding western dairies under current management conditions, are far from desirable. Reproductive ultrasound is another tool available to dairy practitioners to improve reproductive performance. Due to practical limitations and the cost of ultrasound equipment, dairy practitioners have been slow to adopt this technology. Recent significant improvements and less expensive portable ultrasound units make practicality and cost less of a concern.

Reproductive ultrasound in the dairy industry is most often thought of as a means of early pregnancy diagnosis.[2] With significant decreases in conception rates and failure to observe visual estrus, being able to identify open cows as soon as possible and then initiate a timed breeding program can improve pregnancy rates (percent of eligible cows becoming pregnant over a 21-d period) by increasing the heat detection rate. However conception rates to synchronization programs still are below desired levels and keep pregnancy rates low. Currently in the western U.S. dairy industry, the majority of cows found not pregnant at pregnancy diagnosis are immediately started on a synchronization program or in some cases the program is initiated a week prior to pregnancy diagnosis as because GnRH has not been shown to affect pregnancy.[3] Upon being diagnosed not pregnant, cows are then given prostaglandin and continued in the synchronization program. Both of these strategies initiate synchronization programs no matter where the cow is within her estrous cycle.

It has been shown that conception rates vary greatly depending upon when synchronization programs are started in relation to the cow's estrous cycle. Most studies find that the Ovsynch timed AI program results in the greatest conception rates when initiated between d 5 to 12 of the estrous cycle.[4-6] Research conducted in Colorado on commercial dairies found that a large percentage of cows at first service or diagnosed not pregnant are not within

this optimal 5 to 12 d window to start synchronization programs. When these cows are allowed to continue through programs, conception rates are extremely low. Not only are these non-synchronized cows outside the d 5 to 12 window, but a large percentage are acyclic, have cystic ovaries, contain dead fetuses, or have pyometra, which make them poor candidates for enrollment in a synchronization program.[7]

An advantage of ultrasound over rectal palpation is the ability to completely assess ovarian structures and better predict when cows fall into the optimum period to initiate or continue in synchronization programs. Most important in predicting the stage of a cow's estrous cycle is identifying the presence of an active corpus luteum. Ultrasound has been found to be far superior to rectal palpation in predicting an active corpus luteum and high progesterone levels.[8,9] By using ultrasound, cows can be assessed and synchronization programs can be modified when cows fail to respond to the first GnRH injection. Combining ultrasound with a synchronization program can be a powerful management tool to maximize not only heat detection rates, but also conception rates resulting in improved overall pregnancy rates.

Ultrasound can also be used as a heat detection aid, especially for dairies that utilize tail chalk. Accuracy of heat detection when using tail chalk can vary, and often diestrus or even pregnant cattle are bred. By using the ultrasound, cows with an active corpus luteum can be avoided, and only cows appearing in estrus (hyperechoic uterus, ovulatory follicle and no corpus luteum) will be bred.

However, western U.S. dairies are also experiencing an increased cow-to-employee ratio, which makes increasing the level of reproductive management a challenge. In order to implement this reproductive management system, dairies must increase the number of times cattle are handled for ultrasound examination, injections, and artificial insemination. Only dairies capable of handling large numbers of cattle in a short period of time will be able to incorporate these protocols effectively. Currently most large dairies in the western U.S. utilize lock-ups to handle cattle. This system requires that cows actively enter and lock themselves, which is not always ideal. Almost 100% of the time this system results in some loose cattle requiring time to find and then restrain.

Management/palpation rails were created and gained popularity with the advent of bovine somatotropin. If designed properly, this system can be very efficient in working large numbers of cattle in a short period of time. Currently on one client's 10,000 cow dairy, our practice incorporates ultrasound exclusively and provides 100% TAI. Cattle are handled in a roofed double-40 palpation rail. With three ultrasonographers, our practice scans 300-450 cows/hour, and using multiple AI technicians we inseminate approximately 250 cows/hour. By ultrasounding, injecting, and inseminating hundreds of cows in a very short time period, we maintain the time intervals for synchronization and thus maximize conception rates. Only producers willing to restructure their reproductive management and build facilities around it will be able to implement these protocols effectively.

Conclusion

Cows have changed so much reproductively over the last 20 years that it takes greater attention to detail and a better understanding of reproductive physiology to get cows pregnant. Producers are reluctant to truly admit that cows have changed. They still want to manage cows reproductively as if they were in the 1980s. Acknowledging that cows are not the same and then implementing intensive management protocols to overcome these obstacles is critical to improving reproduction. Everything we thought we knew about the reproduction of lactating dairy cows has to be reconsidered. Thinking outside the box and applying new strategies to reproductive management can pay big returns. Incorporating ultrasound intensively into TAI and estrus detection programs can improve rates dramatically, but requires a different mindset for managing cows.

Managing reproduction is unlike any other task on the dairy. To succeed, attention to detail has to border on obsessive-compulsive. Synchronization programs require attention to detail, strict adherence to injection schedules, and proper AI technique. Managing high numbers of cows within these programs also requires strict data entry and retrieval within dairy software programs. So, no matter how well the cows are started on these programs, if compliance is inadequate, results will be inferior. Failure to strive for perfection in every aspect of reproductive management is doomed to poor results. Finding people that have the work ethic and are knowledgeable of reproduction is exceedingly difficult for producers.

Other tasks within the dairy industry have been contracted over the years, such as crop production and calf/heifer production. By designing a veterinary practice around the management of reproduction and employing highly skilled individuals to conduct all aspects of reproduction from ultrasound, injections, and AI to data entry/retrieval reproduction can be improved. The greatest chance of reproductive success occurs when incorporating ultrasound into both synchronization and heat detection programs on dairies.

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Managing the problem beef embryo donor

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Abstract

Defining the problem beef embryo donor and the methods of managing each problem can differ with each donor presented. To manage the problems presented to the practitioner the answer may include any one, or a combination of conservative medical or surgical solutions. The donor's life and physical health are rarely at risk when dealing with embryo production issues. Therefore, the treatment protocol should be based on the possibility of increasing her embryo production, and whether the expense involved is justified.

Keywords: Problem donor, management, treatment, expense

Introduction

What is a problem donor? The argument can be made for defining the problem donor as that animal with physical or reproductive problems that deny her any possibility of producing viable embryos on the day of embryo collection or one that just gives fewer embryos than the average. In embryo transfer practice the definition of a problem donor is primarily dependent upon the owner of the cow. One owner's definition of a problem donor can be another owner's definition of acceptable. The financial as well as emotional investment is weighed in defining whether or not the individual donor has a problem. If the beef donor is the son's or daughter's present or past show heifer, or if she is the dam of the steer that won a major show, then her status as a problem donor may differ when compared to the embryo donor that is used only to fill an embryo purchase quota. Emotional involvement and potential windfall gain, as compared to economic returns based on embryo production per collection can drastically alter the definition of the problem donor. The difficulty is to arrive at a definition of the problem beef donor that allows the embryo transfer practitioner to make consistent recommendations for managing a problem while at the same time taking into account the variations of expectations that result from each individual case.

Donor Embryo Production

The average number of viable embryos recovered per collection from stimulated beef donors as reported by the American Embryo Transfer Association (AETA) in 2007 was 6.52.[1] In 2006 embryo production was 6.50[1] and review of the past averages reveals there have not been appreciable changes since the reporting began. This gives a baseline for the discussion with the owner, and allows him or her to make treatment decisions based on the animal's peers.

Donor Management Problems

The problems that are routinely seen in the beef donor that result in less than expected results can be categorized into problems associated with the owner, the practitioner, and those directly related to the animal. The owner's problems can be the easiest or the most difficult to solve. Many times the owner's problem is solved through education. Helping the owner understand what is and is not a problem is crucial. If he/she has been misinformed by breeders, journals, or other sources about expectations of embryo production a quick review of the national averages[1] will resolve the problem. A second owner-related issue that can lead to a donor problem is the use of frozen semen from a sire with poor fertility. The pressure to use the new up-and-coming sire or the bull that sired the high selling lot at the last breed sale may take precedence over possible fertility issues. Even if a history of fertility problems with the sire in question can be documented the decision to change sire choice to one with good fertility can be a difficult task.

The second group of problems that can negatively affect the donor's production are problems related to the practitioner. The donor problems that can arise from the practitioner range from inexperience, substandard donor protocols, inadequate embryo equipment, handling facilities, weather, and cattle breed and age variations to name a few.

The third category of problems is that linked directly to the animal herself. These decreases in production can be related to age, breed, malnutrition, obesity, disease, lameness, genetic or congenital defects, reproductive disease, trauma, or dystocia.

Solutions and Treatments

To assess and devise a management plan for the many causes of decreased production in the donor requires addressing all three categories of problems.

Owner-related problems

Owner education is the key to an understanding of embryo transfer goals. It is not always easy for the owner to hear that not all donors give "25 number 1 eggs" at every collection, but it is better to give realistic

goals to the owner before the expenses and time required for a successful or non-successful superovulation and collection are incurred. Embryo production can be extremely high but there are many more donors that give no viable embryos in a collection as opposed to those that produce twenty five or more that all grade as quality score one.[2] Sire selection is a difficult decision for the owner. In a superovulation program it should be given considerable attention. With embryo production the practitioner's primary goal, the most consistent sire selection criteria available is to use semen with known collection results. Whenever possible obtain frozen semen that you have personal experience using in your practice. The same semen from the same cane from the same bull taken from the same ranch nitrogen storage tank is the best way to insure consistent sire results. Keep detailed records on every sire and refer to the results when discussing sire assignments with the owner. He will make the final decision, but your knowledge of previous sire success or failure can be a big influence.

Practitioner-related problems

The failure to reach production goals because of mistakes, inexperience, and lack of specific reproductive knowledge by the practitioner is not a minor problem. If the practitioner can not devote a large percentage of their time to embryo work they will not know what equipment is necessary in specific situations, what programming differences are needed related to breed and age, and how to deal with individual animal variations to obtain the maximum production at each collection. The virgin heifer is a good example. She can be a productive embryo producer if managed properly. By monitoring the estrous cycle, decreasing the total amount of FSH, minimizing external stresses, and using specific collection equipment and techniques a practitioner can increase the percentages of success with young heifers. If the virgin heifer is managed as an adult the results will be disappointing. The opposite end of the spectrum is the obese older American breed (Brahman influenced) donor. Finding the proper stimulation protocols, and having the equipment and ability needed to effectively collect this cow type is quite a challenge. There are 1800 pound Beefmaster donors that require less FSH than a virgin heifer, have an extremely large cervix and ovaries that are larger during postpartum anestrous than most bovine superstimulated ovaries. The list of normal variations in the beef donor is lengthy but the point is, the practitioner must be knowledgeable of the different skills, techniques, and donor requirements needed to handle the variations as they are presented.

Animal-related Problems

Many of the donor problems related to the cow can be solved with time. Very commonly a young normal heifer is presented as a potential donor and has not yet reached puberty. A post partum cow or a thin nursing cow is presented and is not yet cycling. These are not true problems but normal occurrences in the cow's life. A group of reproductive problems that can be treated either conservatively or aggressively, depending on the specific problem's effect on embryo production are urine pooling, pneumovagina, and vulvar tears. My approach to this group of problems is conservative as long as no uterine involvement is detected. I have adopted this approach because of the examination of recipient pregnancy records. Notations on the transfer record for each of these three problems were made every time a recipient was presented for transfer and the pregnancy results were examined. The pregnancy rates of those affected recipients with urine pooling, pneumovagina, or vulvar tears, but no indication of uterine involvement, did not differ from the non-affected recipients. The donor with vulvar tears that allow vaginal fecal contamination and the urine poolers that show signs of metritis are surgically repaired. The true problems that affect production are often times problems that cannot be reversed. Blockage of the oviduct (whether cystic or caseated), chronic mucometra, and severe uterine/ovarian adhesions are a few examples. These problems can be handled in one of two ways, either sell the donor or refer her to an in-vitro fertilization (IVF) clinic. The options are discussed with the owner as to the cost of the procedure and expected results. If the value of the donor's offspring warrant the expense then IVF is a good option.

Conclusion

I hope I have made you aware that most of the problem donors that are presented can be managed conservatively with success. The beef cow is one of the most reproductively resilient animals veterinarians will ever encounter. The nutritional, physical, infectious, genetic, and iatrogenic stresses that the cow can withstand and still be reproductively prolific is quite remarkeable.

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Bovine richomoniasis: a review

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Abstract

Trichomoniasis is a bovine venereal disease that causes substantial economic losses. Bulls serve as asymptomatic carriers for the protozoan *Tritrichomonas foetus,* whereas infection in females may result in early embryonic death, abortion, pyometra, fetal maceration, or infertility, all of which negatively influence the profitability of a cattle operation. When allowed adequate recovery time following infection most females mount an immune response and return to normal reproductive status. However, the male can remain infected and remain a risk to a producer's breeding program. Currently no legal treatment for this disease exists in the United States therefore veterinarians and cattle producers must focus on preventive management and surveillance measures such as testing, identification and removal of positive animals. Understanding the pathogenesis, prevalence, economic impact, and diagnosis of trichomoniasis will assist with implementation of appropriate prevention and control programs. This paper reviews the pathogenesis, prevalence, economic impact, and diagnosis of trichomoniasis in cattle, as well as common guidelines for the prevention and control of trichomoniasis.

Keywords: Trichomoniasis, *Tritrichomonas foetus*, epithelial crypts.

Introduction

The bovine venereal disease trichomoniasis is caused by the protozoan *Tritrichomonas foetus (T. foetus).* Bulls serve as asymptomatic carriers when the organism colonizes the epithelium of the penis or prepuce with no clinical signs. Following coitus or artificial insemination with contaminated semen females develop uterine infections that may lead to early embryonic death, abortion, fetal maceration, pyometra or infertility. This manuscript reviews the pathophysiology of bovine trichomoniasis as well as common guidelines for diagnosis

Venereal Disease

T. foetus is an obligate parasite of the bovine reproductive tract. Similar to most venereal diseases, the male is an asymptomatic carrier while the female suffers identifiable consequences of infection. *T. foetus* in bulls localizes in the smegma (secretions) of the epithelial lining of the penis, prepuce, and distal urethra.[1] The organism does not invade the epithelium, and therefore does not invoke an immune response in the bull.[2] *T .foetus* causes no penile or preputial lesions and does not affect libido.[3,4] There are no observable changes in semen quality attributable to the organism's presence, however in a recent study by Benchimol, et al, exposure to *T. foetus* resulted in decreased spermatozoal motility, agglutination of sperm cells, and eventual phagocytosis.[5-7] The only clinical sign that may be observed in an infected bull is a mild transient preputial discharge during the first two weeks of an infection.[4]

Infection in young bulls (less than 3-4 years of age) is purportedly most often transient, with disease transmission only occurring if sexual contact with a non-infected cow occurs within minutes to days following breeding of an infected cow.[8,9] Studies by Morgan[8] and Clark[9] indicate that clearance of the organism in a young bull is possible within 20 minutes following breeding an infected cow. Transmission of *T. foetus* by a young bull is therefore likely to be a passive, mechanical transmission that differs from transmission associated with a chronically infected older bull.

T. foetus infection in the cow occurs during coitus with an infected bull. The organism transverses the cervix and colonizes the entire reproductive tract within 1-2 weeks,[10] and as the organism multiplies in the uterus it can cause death of the embryo or fetus, most commonly between gestational days 15 to 80.[11] Pyometra and abortion are often the first physical signs of trichomoniasis noticed in a herd, but these signs occur in fewer than 5% of infected animals.[12] Infertility due to embryonic death is the most economically damaging clinical sign and occurs in a larger percentage of infected cows. An affected cow's interestrus interval is usually prolonged because the embryonic loss typically occurs after maternal recognition of pregnancy (days 15-17 of gestation).[10] Unlike the bull, the cow typically mounts an effective immune response to *T. foetus*,[4] but the time it takes to clear *T. foetus* from the cow's reproductive tract is quite variable. Primary infections may be cleared from the reproductive tract in as little as 95 days[13] or as long as 22 months.[8] Subsequent infections are cleared in about 20 days, indicating an anamnestic response.[9] Immunity does not persist, however, and the anamnestic response is only significant if re-infection occurs within about 15 months of the primary infection.[14,15] A cow in a herd with a long breeding season could therefore become pregnant and infected with *T. foetus* early in the breeding season, lose that embryo, be infertile for several months, clear the initial *T. foetus* infection, rebreed, conceive, and carry a calf to term as a result of temporary immunity. The result is that more cows will calve later in the calving season than desired, and there is a resultant wide variety in weaning weights rather than just a reduced calving percentage. The later-born calves are

then marketed at lighter weights, or the cattle producer will incur increased feeding costs to achieve a desired market weight. In either case the cattle producer will sustain substantial economic losses.

Economic aspects

Economic losses due to venereal disease result from culling and replacement of infected animals, a decreased percentage of cows calving or calving later than desired with subsequent reduced calf crop and lower weaning weight caused by failure or delay of conception.[14] Fitzgerald, et al[16] estimated in 1958 that each infected bull in a large herd was responsible for an $800 loss per year. Wilson, et al[17] estimated a $2.5 million annual calf loss in 1979 due to trichomoniasis in Oklahoma replacement heifers. In 1986, Fitzgerald estimated that the total economic impact in the USA was $65 million annually.[18] In a 1991 study, Speer, et al estimated that annual losses could reach near $650 million.[19] Recently the state of Louisiana estimates that current losses exceed $100 million for that state alone, so the economic loss is likely considerably greater than earlier studies.[20]

During the 1990's the Idaho legislature approved statutes that prescribe regulations for identifying and eliminating *T. foetus* bulls within the state and for importation of bulls into that state.[21] Since that time other states have adopted similar legislation and currently the states of Nebraska, North Dakota, South Dakota, Montana, Wyoming, Idaho, Washington, Oregon and Utah require bulls be test negative for trichomoniasis before being transported into the state, sold, or used on public land.[22-29] The Texas Animal Health Commission has recently passed similar requirements[30]. These regulations reflect the growing concern for control of this bovine venereal disease which is so economically important in the United States.

Diagnosis of Bovine Trichomoniasis

Diagnosis of *T. foetus* has traditionally relied upon microscopic identification of key morphological characteristics in preputial smegma or cervicovaginal mucus (CVM) incubated in various culture media. Such characteristics include three anterior flagella, one posterior flagellum, and an undulating membrane resulting in a jerky movement pattern. However, accurate microscopic identification of *T. foetus* can be complicated by the presence of other trichomonadid protozoa.[31-35] Contamination of the preputial orifice, prepuce, or penis with fecal material probably explains the presence of these opportunistic trichomonads. Several non-pathogenic protozoa are normal inhabitants of the bovine gastrointestinal tract,[36-38] and therefore proper cleaning of the preputial orifice and proper sampling techniques are critical to avoid fecal contamination of diagnostic samples. None of the contaminating trichomonads, however, results in reproductive pathology in cows or bulls.[39] Therefore, research has recently focused on molecular-based assays to accurately differentiate *T. foetus*[33,40-42] from other trichomonads. Given the lack of legal therapy for bulls infected with *T. foetus* in the United States the only reasonable course of action is to slaughter an infected bull. It is therefore imperative to correctly identify *T. foetus*-infected bulls and not misdiagnose based on the presence of non-pathogenic fecal trichomonads.

At present, molecular-based assays are most commonly used as confirmatory tests for bovine trichomoniasis because of the relatively low cost of *in vitro* cultivation compared to molecular-based assays. However, molecular-based assays are currently very effective in diagnosing human trichomoniasis caused by *Trichomonas vaginalis*, with a sensitivity of 95% and a specificity of 98%.[43] It is therefore very likely that in the future the preferred diagnostic test for bovine trichomoniasis will be a molecular-based assay, and some researchers have already advocated their use as an independent diagnostic test for bovine trichomoniasis.[44,45]

Sampling techniques for detection of trichomoniasis in the male

Several sampling techniques are utilized for obtaining diagnostic specimens in the bull including: 1) a swab technique;[46] 2) a dry pipette technique;[9,47] 3) a wet pipette technique;[48] and 4) the douche technique.[48] Fitzgerald, et al compared the swab and pipette techniques and reported that the number of parasites recovered via the swab technique is only 20% of the number of parasites recovered via pipette scraping.[49] The swab technique is therefore rarely used in the United States. The dry pipette technique is one of the most common sampling methods in the U SA, while the douche method is the preferred technique in Europe.[47] Schönmann, et al reported that the two methods are not statistically different.[47]

Regardless of technique used, it is generally recommended that bulls be sexually rested 1-2 weeks before testing for *T. foetus*; otherwise, false-negative results are more likely because breeding mechanically removes many of the organisms from a bull's penis and prepuce. Given the sensitivity of *T. foetus* cultures, false-negative results are also possible even if a bull has been sexually rested. Only with three negative tests at weekly intervals (Figure 1) can a veterinarian or producer be 99% sure that a bull is *T. foetus* negative.[50]

	Result	Sensitivity (in series)
First test	Negative	80%
Second test (one week later)	Negative	96%
Third test (one week later)	Negative	99

Figure 1. Sensitivity (in series) of *T. foetus* cultures.[50]

Sampling techniques for diagnosis of trichomoniasis in the female

Researchers investigating diagnostic sampling methodologies for *T. foetus* have focused primarily on optimizing sample collection and culture from bulls because of their propensity to develop chronic infections. The technique most commonly used to sample female cattle for *T. foetus* is a dry pipette technique.[48] An infusion pipette is used to aspirate CVM from the vaginal fornix or near the external cervical os. Alternatively, in the case of a post-coital pyometra, an infusion pipette can also be used to aspirate some of the content of the uterus. Either sample is then examined directly or placed into appropriate culture medium. Culturing *T. foetus* from CVM has a reported sensitivity of 58 to 75%.[51] Samples can also be evaluated with appropriate molecular-based assays.

In vitro culture of *Trichomoniasis foetus*

Direct microscopic examination of specimens for *T. foetus* may be diagnostic, but a far more sensitive method for the detection of *T. foetus* is *in vitro* culture of preputial smegma in a selective nutrient medium for up to a week.[51-53] *In vitro* culture allows the proliferation of *T. foetus* to more readily detectable levels. All cultures containing organisms resembling *T. foetus* should be confirmed with appropriate molecular-based assays to avoid false-positive results due to fecal trichomonad contamination of culture media.[31,32,54] Alternatively, samples may be submitted directly for molecular-based evaluation. If polymerase chain reaction-based evaluations are not available, a current study by Corbeil, et al suggest that immunofluorescent assay may be useful in the diagnosis of T. foetus.[55]

In vitro culture media

Various culture and transport media systems have been used including Kupferberg medium and broth, Claussen's medium, Sutherland medium, trypticase-yeast extract-maltose (TYM) medium, Diamond's medium, and most recently the InPouch® TF (BioMed Diagnostics, White City, OR, USA) *Tritrichomonas foetus* culture pouch. *In vitro* cultivation using either Diamond's medium or the InPouch® TF is currently the most common method used to diagnose *T. foetus* in the United States. Both culture systems are fairly equal in sensitivity.[47,56-58] However, the InPouch® TF is somewhat more convenient than Diamond's medium.[59] The InPouch® TF has a 12-month shelf-life at room temperature, compared to a much shorter refrigerator-life for Diamond's medium. Also, the plastic pouch design of the InPouch® TF is less likely to break or leak than tubes containing Diamond's medium. Unfortunately, the InPouch® TF is more expensive than Diamond's medium.

For many years, cultivation of microorganisms with motility and morphology resembling *T. foetus* in either the InPouch® TF or Diamond's medium was considered to be 100% specific. However, accurate microscopic identification of *T. foetus* has since been shown to be complicated by the presence of other contaminating trichomonadid protozoa. All cultures containing organisms resembling *T. foetus* should therefore be confirmed with appropriate molecular-based assays, or samples should be submitted directly to a laboratory for molecular analysis. Contact the laboratory prior to sample collection to verify the appropriate transport medium.

Treatment of cattle infected with *Tritrichomoniasis foetus*

One of the complicating factors associated with bovine trichomoniasis is that there are currently no effective treatments with U.S. Food and Drug Administration approval. Historically, the most successful treatment for bulls with trichomoniasis involved systemic treatment with nitromidazole derivatives.[51,60-62] Despite its effectiveness, the use of nitromidazole derivatives is now illegal in food-producing animals in the U.S. because of their mutagenic and carcinogenic properties, and no alternative treatments are available. However, a recent study by Carvalho, et al[63] found that *T. foetus* exposed *in vitro* to mebendazole resulted in internalization of the flagella, disruption of the nucleus, and cytoplasmic vacuolization. These findings suggest new possibilities in the treatment of trichomonasis. Still, the lack of effective approved therapies for bovine trichomoniasis emphasizes the need for appropriate preventive and control measures.

Prevention and Control of Bovine Trichomoniasis

Preventing the introduction of *T. foetus* into a cattle herd and controlling trichomoniasis in an infected herd follow many of the same management strategies and to a large extent focus on herd biosecurity. Ideally, every cattle operation should focus on preventing the introduction of *T. foetus*.

Recommended practices to prevent the introduction of *T. foetus* into a cattle herd include:

1) When possible, avoid grazing cattle on public lands where both bulls and cows have a much greater risk of exposure through coitus with other *T. foetus*-infected animals.[64]
2) Utilize artificial insemination when possible
3) Cull all open cows and heifers.
4) Control animal movement into a herd. Maintain good fences to prevent *T. foetus*-infected animals from inadvertently entering a herd, or to prevent uninfected animals from temporarily entering a *T. foetus*-infected herd and then returning with *T. foetus* to their uninfected herd of origin.
5) Purchase virgin bulls and heifers as replacements. Buying older bulls and cows as replacements greatly increases the chance of purchasing a *T. foetus*-infected animal. While older bulls are much more likely to become chronically infected with *T. foetus* than cows, a small percentage of cows will also become chronically infected.
6) Test bulls for *T. foetus* at least once before introducing them into a new herd
7) The test should be performed after two weeks of sexual rest. Ideally, a bull should have three negative cultures at weekly intervals.
8) Maintain as young a bull battery as possible. Older bulls are considered more likely to develop chronic *T. foetus* infections. However, any bull exposed to *T. foetus* in a natural breeding situation is capable of becoming chronically infected, regardless of age.
9) Breed purchased cows and heifers in a separate herd, and cull all open animals. Ideally, continue to keep the pregnant animals segregated from the rest of the herd through the next breeding season.
10) Consider immunization against *T. foetus* in high-risk herds.

Recommendations for control of trichomoniasis in an infected herd includes:

1) Test and cull all infected bulls. Infected bulls should be sold for slaughter only.
2) Decrease the number of bulls per breeding unit. Single-sire herds offer the lowest exposure potential. However, single-sire units may not always be practical.
3) Reduce the average age of the bull herd. Older bulls are considered more likely to develop chronic *T. foetus* infections. However, any bull exposed to *T. foetus* in a natural breeding situation is capable of becoming chronically infected, regardless of age.
4) Test bulls for *T. foetus* at least once before introducing them into a new herd. The test should be performed after two weeks of sexual rest. Ideally, a bull should have three negative cultures at weekly intervals.
5) Utilize artificial insemination when possible
6) Reduce the breeding season to 60-90 days and cull all open cows and heifers. If there are too many open cows for culling to be economically feasible, then at least these animals should be separated into a high-risk herd. A long breeding season not only allows propagation of *T. foetus*, but it may also hide production losses due to reduced weaning weights because of delayed conception.
7) Culture all cases of pyometra diagnosed in cows or heifers during pregnancy examinations.
8) Submit all aborted fetuses and placental tissue to a diagnostic laboratory.

Immunization against *T. foetus* is an extremely important management tool for herds infected with *T. foetus*. Research trials clearly demonstrate the benefit of *T. foetus* vaccination.[64-66] TrichGuard® (Fort Dodge Animal Health, Fort Dodge, IA, USA) and TrichGuard® V5L (Fort Dodge Animal Health) are currently the only *T. foetus* vaccines available in the United States. The vaccines require an initial subcutaneous dose followed by a booster dose two to four weeks later. The second injection should precede the breeding season by four weeks. Annual revaccination four weeks prior to the breeding season is recommended.

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Ecbolic and tocolytic agents in bovine reproduction

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Abstract

The use and efficacy of ecbolic and tocolytic agents to treat and manage reproductive conditions in the bovine have been controversial topics at times. This is in part due to our limited understanding and lack of research with regard to the pregnant and post partum uterus in the cattle. This article reviews the anatomy and physiology of the bovine uterus and research evaluating the use and efficacy of various ecbolic and tocolytic compounds.

Keywords: Bovine uterus, ecbolic, tocolytic

Introduction

Ecbolic agents have been administered in an attempt to treat and prevent many reproductive conditions in the periparturient cow which most commonly include retained fetal membranes, post-partum endometritis and metritis. Tocolytic agents have been used to inhibit uterine contractility in a variety of clinical situations which include delaying parturition and facilitating obstetrical manipulations. To further understand the uses and possible roles of ecbolics and tocolytics in bovine practice, one must first understand the anatomy and physiology of the uterus and the pharmacology of these agents.

Anatomy and physiology of the uterus

The uterine wall is composed of three layers. The innermost layer, the endometrium, lines the lumen of the organ and consists of columnar epithelium and underlying stromal tissues. The middle layer, the myometrium, which consists predominately of smooth muscle cells, also contains blood and lymph vessels, nerves, immune cells, and connective tissue. The outer layer, the serosa, is a thin layer which covers most of the uterus and is composed of mesothelial cells. The individual smooth muscle cells of the myometrium are the physiologic units of uterine contraction. The muscle fibers of the outer longitudinal layer are arranged parallel and those of the inner circular layer are arranged concentrically around the long axis of the uterus. Advancing gestation is accompanied with hypertrophy and hyperplasia of the uterine smooth muscle. The increase in size can be up to three- to fivefold by the end of gestation.[1,2]

Smooth muscles are innervated by the sympathetic nervous system and have α and β receptors. The α receptors are responsible for muscular contractions while the β receptors are responsible for relaxation. The β_1 receptors are confined to the heart, adipose tissue, and small intestine. Stimulation of β_1 receptors leads to increased cardiac automaticity, positive chronotropic and inotropic effects, and elevated free fatty acids. The β_2 receptors are found in the smooth muscle of the uterus (myometrium), vascular smooth muscle, and bronchioles. Stimulation of β_2 receptors causes relaxation of the uterus, vasodilation, and bronchodilation. Activation of these β_2 receptors leads to an elevation of in cAMP, mediated through adenylate cyclase. These increased levels of cAMP prevent myosin light-chain kinase (MLCK) activity through both decreased phosphorylation and inhibition of release of stored intracellular calcium thus inhibiting uterine contraction.[1,2]

Uterine contractility

The contractile activity of the uterus is directly related to the electrical activity in the smooth muscle cells. This activity is characterized by cyclic depolarization and repolarization of the plasma membrane and action potentials. Contraction of the smooth muscle cells occurs by the interaction of the myofilaments which are composed of myosin and actin. The sarcoplasmic reticulum also plays a key role as it is the site of calcium storage. In the resting stage, the intracellular calcium in the smooth muscle is low. Contraction of the smooth muscle is preceded by an increase in free intracellular calcium levels. Calcium ions bind with calmodulin which then activates myosin kinase. Myosin kinase in turns phosphorylates a myosin head. The phosphorylated myosin head then binds with an actin filament, thus inducing smooth muscle contraction.[2]

During pregnancy, the uterine smooth muscle is relatively quiescent, displaying weak, localized, and poorly coordinated contractions. In contrast, during parturition, the contractions are forceful, sustained, regular, and well-synchronized. Factors responsible for initiating the process of parturition include 1) an increase in the number of gap junctions which allows cell coupling and interactions, 2) decreased production of nitric oxide thereby inhibiting uterine relaxation, and 3) stretch of the myometrium which enhances contractility.[2]

Ecbolic agents (oxytocics)

Ecbolic agents or oxytocics are compounds that hasten uterine evacuation by stimulating uterine contractions of the myometrium. The best known and most widely used ecbolics are oxytocin and prostaglandins (PG). Ecbolics have been used for the treatment of retained placentas and for the treatment and prevention of metritis.

Oxytocin

In 1906, Sir Henry Dale reported that an extract of the neurohypophysis had oxytocic effects. The use of this extract to induce labor was first reported as early as 1911. The name given to this compound was oxytocin, derived from the Greek word meaning "swift birth." Oxytocin is a neuropeptide hormone produced by magnocellular cells in the hypothalamus, transported to the posterior pituitary via axons, and stored and secreted by the posterior pituitary. Another major function of oxytocin in mammals is stimulation of milk letdown from the mammary glands. In the 1950s Du Vigneaud and others established the structure and synthesized oxytocin. Oxytocin was the first peptide to be synthesized.[3-5]

Oxytocin is a potent stimulus for uterine contractions. The action of oxytocin is mediated by binding to specific oxytocin receptors present in the uterus when the uterus is under the influence of estrogen. Thus oxytocin is considered to be effective in causing uterine contraction and uterine evacuation if administered within 48 to 72 hours postpartum. The binding of oxytocin to its receptors causes an increase in intracellular calcium which results in contraction of myometrial cells from the uterus. Oxytocin has three specific actions on the uterus which include 1) induction of myometrial contractions, 2) release of $PGF_{2\alpha}$ from the endometrium, and 3) release of PGE_2 from cervical mucosa. Most formulations of oxytocin contain 20 United States Pharmacopeia (USP) units/mL with package inserts recommending up to 100 USP units. This dosage recommendation seems high considering that 1.0 IU of oxytocin achieves physiologic levels comparable to those seen during milking. Thus, an oxytocin dosage of 10 IU is a supra-physiologic dosage.[6]

Prostaglandins

Prostaglandins were discovered in the early 1930s as substances present in human seminal plasma that induced, and sometimes relaxed, uterine muscle activity *in vitro*. Von Euler named these substances prostaglandins believing that they came from the prostate gland. Although, it was later discovered that the seminal vesicles were the major source of prostaglandins; thus, prostaglandins is really a misnomer. In 1957, Bergstrom and Sjovall reported the first isolation of prostaglandins and determined their structures. By the 1960s, Sammuelsson and colleagues began describing the prostaglandin metabolic pathways. In 1971, Vane reported that aspirin inhibited PG production and later showed that non-steroidal anti-inflammatory drugs acted via PG inhibition. Bergstrom, Sammuelsson, and Vane were awarded the Nobel Prize in 1982 for their work with PGs.[5]

When biologically active PGs are released into the blood they are metabolized by enzymes in the liver, kidney, and particularly the lung. After one passage of $PGF_{2\alpha}$ through the lungs, over 90% of the PG is metabolized. This is one reason why most PGs have very short half-lives in blood (<1 min). In addition to the lung, the placenta also has very high concentrations of PG dehydrogenase. Thus, it is very unlikely the biologically active PGs can cross the placenta and affect the fetus. Prostaglandins are capable of causing muscle relaxation and muscle contraction depending upon the branch of the receptors (relaxant or stimulatory receptors). The relaxant receptors act via adenylate cyclase to elevate levels of intracellular cyclic adenosine monophosphate (cAMP) and phosphokinase A (PKA) activity. This in turns relaxes smooth muscle via elevated cAMP which induces sequestration of intracellular calcium. The stimulant receptors act via enhancing intracellular phosphokinase C (PKC) activity and intracellular calcium. The activity through this receptor stimulates smooth muscle contraction by elevating intracellular calcium levels. This discussion will focus on the stimulatory effects of PGs, specifically $PGF_{2\alpha}$, on the uterus.[5,6]

During the immediate postpartum period, serum concentrations of $PGF_{2\alpha}$ and its metabolites are elevated. These elevations are thought to facilitate uterine involution. Prostaglandin $F_{2\alpha}$ has many actions, including luteolysis, stimulation of myometrium, and constriction of blood vessels. In addition, PGs also have a relaxant effect on the cervix. Thus, $PGF_{2\alpha}$ is used to treat a variety of medical conditions. These conditions include 1) induction of parturition, 2) lysis of corpus luteum (CL) for cases of pyometra, 3) lysis of CL for management of the estrous cycle, 4) induction of abortion, and 5) evacuation of the uterus in cases of metritis or endometritis. Prostaglandins can be used to induce parturition and abortion by its uterotonic effect and by its luteolytic effect. Prostaglandins used in veterinary medicine include cloprostenol sodium, dinoprost tromethamine, fenprostalene, fluprostinol sodium, alfaprostol, and luprostiol.[6]

Cloprostenol sodium. Cloprostenol (Estrumate®, Intervet/Schering-Plough Animal Health Corp., Summit, NJ, USA) is a powerful luteolytic agent and causes rapid regression of the CL and arrests its secretory activity. Cloprostenol is available in a concentration of 250 mcg/mL and is a PG analog that is administered by intramuscular injection for all indications in both beef and dairy cattle. Cloprostenol is used in beef or dairy cattle to induce luteolysis. It is recommended by the manufacturer for unobserved or undetected estrus in cows cycling normally, pyometra or chronic endometritis, expulsion of mummified fetus, luteal cysts, induced abortions after mismating and to schedule estrus and ovulation for controlled breeding.[7]

Dinoprost tromethamine. Dinoprost tromethamine (Lutalyse®, Pfizer Animal Health, New York, NY) is the naturally-occurring $PGF_{2\alpha}$ as the tromethamine salt. Each mL contains 5mg of dinoprost and is luteolytic in

cattle at 25 mg (5 mL) administered intramuscularly. Dinoprost tromethamine is labeled for estrus synchronization, treatment of unobserved (silent) estrus and pyometra (chronic endometritis) in cattle; and for abortion of feedlot and other non-lactating cattle.[7]

Other PGs. Alfaprostol and luprostiol are prostaglandin analogs that are used mainly outside of the United States. These drugs have similar effects and uses as the other synthetic prostaglandins.

Xylazine

Xylazine produces uterine contraction by stimulation of α_2-adrenergic receptors in the uterus. The pregnant bovine uterus appears to be more sensitive to xylazine-induced contractility than the non-pregnant uterus, particularly after 270 days of gestation. This enhanced susceptibility may involve hormone-related changes in α-adrenergic receptor populations in myometrial tissue. In one study, administration of xylazine (10mg intravenously) significantly increased uterine motility during late gestation. Thus, the use of xylazine in the last month of gestation is contraindicated in cattle because of the increased tendency for induction of premature parturition. Xylazine has no practical uses as an ecbolic due to its sedative and muscle relaxant properties.[6,7]

Estrogen

Estrogen has been used in an attempt to initiate and/or strengthen myometrial contractions. However, the use of estrogen is controversial. Because estrogen levels normally decrease dramatically once the calf is expelled, it appears that normal uterine involution can proceed without the influence of estrogen in the normal cow. Studies have shown no beneficial effects on the prevention of metritis or reproductive performance, and that the use of estrogen may actually have a negative effect on subsequent fertility.[8] It is believed that contractions induced by estrogen may force septic uterine contents not only through the cervix but also into the uterine tubes which results in severe bilateral salpingitis.[9] Research has shown that estrogen treatment postpartum has a negative impact on uterine motility where the normal uterine contractions changed to a sustained contraction or spasm.[8] In addition, one study demonstrated that the use of oxytocin in an estrogen-primed uterus did not increase the contraction frequency and thus did not enhance the myometrial effect of oxytocin. Thus, scientific evidence does not support the use of exogenous estrogens in the postpartum cow.

Use of ecbolics to treat retained fetal membranes and uterine infections

Two common problems that are encountered in periparturient dairy cattle and occasionally in beef cattle are retention of fetal membranes and metritis. Retained fetal membranes is one of the most important factors that predisposes cattle to uterine infections. Cattle that have retained fetal membranes are six times more likely to develop a uterine infection than are cows without retained fetal membranes. Primary retention of the fetal membranes results from lack of detachment from the maternal caruncles, whereas secondary retention is related to mechanical difficulty in expelling the already detached fetal membranes. Greater than three-fourths of cows expel the placenta by 6 hours post-partum with the majority of the remaining cows expelling the placenta before 12 hours postpartum. Because the incidence of retained fetal membranes and postpartum disease varies with parity, the definition of retained fetal membranes may also be age- or parity-dependent. Suggestions have been made to define retained fetal membranes from 8 to 48 hours; however, 12 hours is widely used to define retained fetal membranes.[9]

Detachment of the placenta in the cow involves separation of the cotyledon villi from the caruncular crypts without tearing of either fetal or maternal epithelia. For appropriate separation of the cotyledon villi from the caruncular crypts, proteolytic enzymes (collagenases) act to open the cotyledon thereby releasing the caruncle. Collagenase activity of cotyledon villi during delivery is increased in healthy cows and decreased in cows with retained fetal membranes. The cellular sources of collagenase and proteolytic enzymes responsible for placental release in the cow are unknown. However in laboratory animals and humans, myometrial cells, fibroblasts, and leukocytes have been identified as sources of collagenase in the uterus. By day 6 postpartum, the caruncle is disorganized; by day 15, caruncles are completely sloughed as a result of necrosis. Retained fetal membranes are detached by caruncle necrosis within 6 to 10 days and not later than 17 days postpartum. The surface of the endometrium is covered by new epithelium by day 26 to 30 postpartum. After placental detachment, uterine involution is completed in about 39 days in normal cows and 50 days in cows with retained fetal membranes. Lack of uterine motility is not considered a reason for primary retention because uterine motility is normal or above normal in cows with retained fetal membranes.[9]

Factors reportedly contributing to the development of retained fetal membranes include periparturient hypocalcemia, dystocia, abortion, twinning, stillbirth, and induction of parturition. There are several management approaches that have been used for cows with retained fetal membranes of which many are controversial and lack scientific evidence to support their use. These options include no intervention, manual removal of the fetal membranes, antimicrobial therapy, and hormone therapy. No intervention in an otherwise healthy cow is a common practice that allows the fetal membranes to liquefy and necrose until they are passed. These cows should be monitored closely for any signs of septicemia or toxemia in which case systemic therapy is necessary. Manual

removal of the fetal membranes was once practiced but has fallen from favor due to the likelihood of causing trauma to the uterine wall, the high incidence of leaving tags of the fetal membranes within the uterus, and iatrogenic contamination of the uterus. Intrauterine antibiotic therapy is beyond the scope of this discussion but will be discussed in a subsequent presentation. Hormone therapy is still one of the most common methods for managing cows with retained fetal membranes.[6,9,10]

The majority of cattle experience bacterial contamination of the uterus at the time of parturition. In the normal cow, the uterus is cleared of this bacterial contamination by four weeks postpartum. When these bacteria are not cleared by the cow's defense mechanisms, a uterine infection ensues. Numerous bacteria have been isolated from the cow's postpartum uterus, some of which may be incidental and not cause problems. Uterine infections are most commonly due to *Arcanobacterium pyogenes*. The gram negative anaerobes *Fusobacterium necrophorum* and *Bacteroides melaninogenicus* are frequently associated with *A. pyogenes*. Other organisms that may be associated with uterine disease in the cow include *Pseudomonas aeruginosa*, staphylococci, hemolytic streptococci, coliforms, etc. *Clostridium* sp. may occasionally infect the uterus and cause a severe gangrenous metritis or tetanus. Uterine infections are associated with retained fetal membranes, dystocia, and delivery of twins. Metritis is the result of severe inflammation involving all layers of the uterus – endometrial mucosa and submucosa, muscularis, and serosa. Metritis usually develops during the first week after calving and is associated with dystocia, retained fetal membranes, and calving trauma. Affected cattle may be septic and present with fever, depression, and anorexia and a copious fetid vaginal discharge may also be present. Endometritis is characterized by inflammation of the endometrium extending no deeper than the stratum spongiosum. Cows with endometritis are usually not systemically ill, and bacteria are usually eliminated after a few estrous cycles. Pyometra is a collection of purulent exudate within the uterus with the persistence of the corpus luteum, and suspension of the estrous cycle. Pyometra usually develops in cows that have their first postpartum ovulation before bacterial contamination of the uterus has been eliminated. The corpus luteum that is associated with the infection persists because intrauterine fluid prevents luteolysis. Thus progesterone persists and suppressed uterine defense mechanisms.[9,10]

Several different hormones have been used in an attempt to manage retained fetal membranes and uterine infections with the ecbolic agents oxytocin and PG being the most common. Oxytocin appears to stimulate myometrial contraction by 1) direct activation of receptors on myometrial cells and 2) indirect stimulation of contraction through the release of stimulatory PGs from the endometrium. Circulating oxytocin binds to myometrial receptors which leads to rapid uterine contraction and an increase in $PGF_{2\alpha}$ levels. It is believed that $PGF_{2\alpha}$ stimulates the release of more oxytocin and also enhances the sensitivity of the myometrium to oxytocin.[3] As little as 2.5 IU of oxytocin intravenously will cause the proximal ends of the uterine horns to respond within 30 to 50 seconds when progesterone levels are low in a cycling cow, and this increase in myometrial activity persists for up to 80 minutes.[11] Studies such as this in cycling cows have supported the idea that the myometrium is only responsive to oxytocin when estrogen is dominant; whether oxytocin is effective in cows with toxic metritis is unclear.[6] One study indicated that as little as 5 units of oxytocin intravenously can initiate a more intense rhythm of contraction in cows with retained fetal membranes.[12] Other studies refute this evidence and suggest that oxytocin was of no benefit to postpartum cows with retained fetal membranes; however, in these two studies a dose of 60 to 100 units of oxytocin was administered which causes a spasm of the uterus versus a progressive contraction.[13-15] It also appears that the traditional dose of oxytocin (40 units) when administered intravenously causes an initial tetanic spasm of the uterus.[16] Most of the studies demonstrating the positive effect of exogenous oxytocin have used the intravenous route of administration instead of the more commonly used intramuscular route of administration. However, one study did show that the myometrial response following administration of 20 to 30 units of oxytocin was similar following administration via intravenous, intramuscular, and subcutaneous routes of administration.[16] A day two to three protocol of repeated 20 unit (1.0 mL) oxytocin injections administered at least three hours apart or three doses evenly spaced between milkings, etc has been suggested.[6] Although the frequent administration with low dose oxytocin appears to be impractical in most situations, it would appear to induce a more physiologic response than current therapeutic protocols which use infrequent administration at supra-physiologic dosages which induce tetanic uterine spasms. The most physiologic uterotonic dose of oxytocin has not been determined.

Despite much research, the ability of exogenous PGs to have a direct effect on periparturient uterine activity in cattle has been a controversial issue among researchers and clinicians. Although a few studies indicate that $PGF_{2\alpha}$ may reduce the incidence of retained fetal membranes, subsequent studies have failed to confirm these results and many report that exogenous PG has no effect. Many of these studies lack sufficient numbers of animals, lack control animals, and used concurrent medications which make interpretation of the results difficult. It appears that suboptimal uterine contraction is rarely the cause of retained fetal membranes in a nontoxic cow. Studies have shown that the presence of retained fetal membranes alone doubles the rate and increases the frequency of uterine contractions. In another study, cows that had evidence of uterine infection (fetid, sanguine-purulent lochia) at up to

day 15 post partum had significantly higher concentrations of PGF metabolite (PGFM) than did cows that had a mucopurulent to purulent lochia.[17] In addition, studies indicate that a single intramuscular injection of PG before the formation of a functional corpus luteum will have no beneficial effect in the post-partum cow.[12,16] Even when the PG dose was doubled, there was still no increase in uterine tone. However, luteolytic doses of $PGF_{2\alpha}$ (25 mg) administered by rapid intravenous injection on day 2 postpartum did cause an increase in uterine contractions.[16] However, by day four postpartum the stimulatory effect was noticeably decreased. Intravenous administration of PG also has significant side effects (dyspnea, salivation, milk ejection, frequent urination) that make it impractical to use particularly in a toxic cow. Only when luteal tissue is present on the ovary is it widely accepted that exogenous administration of PG has a beneficial effect on the postpartum cow. Intramuscular injection of $PGF_{2\alpha}$ may not be uterotonic because the $PGF_{2\alpha}$ is metabolized almost entirely into PGFM upon a single passage through the lungs. Using PG to lyse the CL allows for removal of the immunosuppressive effects of luteal progesterone which may aid in the resolution of chronic postpartum endometritis.[6] Currently, there is no scientific evidence that intramuscular or subcutaneous injections of either natural or synthetic $PGF_{2\alpha}$ aids in the expulsion of retained fetal membranes. In addition, administration of PG during the immediate postpartum period has not been shown to have an effect on the rate of uterine involution.[18]

Some studies indicate that the use of PG may improve overall reproductive performance in cows that are not affected by periparturient diseases. In addition, cows affected with dystocia, retained fetal membranes, or both that were treated with $PGF_{2\alpha}$ early post partum followed by a second treatment 14 days later experienced a higher conception rates to first service than non-treated cows.[19]

Conclusion

Although more research is needed on the postpartum cow uterus, there is no proven scientific evidence that supports the routine use of ecbolic agents as a treatment for the pathologic postpartum uterus. Based on scientific research, exogenous estrogen and PG at published doses appear unable to stimulate the appropriate rhythmic contractions necessary to empty the pathologic post partum uterus. There is some evidence that supports the use of exogenous oxytocin to stimulate uterine contractions that are similar to those contractions observed during stage II of labor. However, these studies used intravenous oxytocin rather than the more common intramuscular route of administration.

Tocolysis

Tocolysis is derived from Greek with "tokos" meaning childbirth and "lysis" meaning capable of dissolving. A tocolytic agent is a compound that is capable of inhibiting uterine contractions. Tocolytics were originally designed for use in human medicine to interrupt premature labor and have, over time, been used more commonly in veterinary medicine. The use of tocolytics to inhibit uterine contraction has a number of potential clinical applications in cattle. Delaying parturition for controlled calving may be useful if parturition were occurring at a time that decreased fetal survival (nocturnal delivery). Obstetrical manipulations such as correction of malpresentation and malposition, repulsion and rotation of the fetus, correction of uterine torsion, ease of extraperitoneal lifting of uterus during cesarean section, and replacement of uterine torsion may be aided by the use of tocolytic agents. Some believe that these drugs may also be useful in the area of embryo transfer.

Tocolytic Agents

A variety of tocolytics have been used for the aforementioned applications to cause uterine relaxation. Ethyl alcohol, magnesium sulfate, progesterone, prostaglandin synthetase inhibitors, calcium channel blockers, epinephrine, and β sympathomimetics have all been used to induce uterine quiescence. However, the unpredictable efficacy and adverse side effects make some of these drugs less acceptable than others for the induction of uterine relaxation.[20]

Ethanol

In the mid 20th century, ethanol was a commonly used tocolytic agent in human patients to halt pre-term labor. It is believed to effectively inhibit the secretion of oxytocin and interfere with prostaglandin synthesis. Ethanol was given intravenously in humans at a rate to maintain a blood alcohol level of 0.9 to 1.6 mg/liter. Of course, side effects were observed which included nausea, vomiting, depression, intoxication of mother and fetus, and acidosis. Research has since demonstrated that ethanol is not effective in delaying parturition.[21]

Magnesium sulfate

Magnesium sulfate has also been used as a tocolytic. Magnesium sulfate is a central nervous depressant which blocks neuromuscular transmission and lowers acetylcholine. In 1959, the tocolytic properties of magnesium sulfate were first described. The exact mechanism by which magnesium sulfate exerts its tocolytic effects is unknown. However, one possible mechanism may be its ability to block nerve transmission and/or by its actions as a calcium antagonist. Magnesium sulfate has been given intravenously as a 10% solution to delay parturition for 24 to 48 hours. Most human studies do not indicate a significant ability for magnesium sulfate to prolong pregnancy.

The use of magnesium sulfate as a tocolytic is no longer recommended due to its lack of effect at preventing preterm deliveries and because of its association with a higher risk of perinatal death.[22]

Progesterone

The actions of progesterone on the pregnant myometrium include relaxation of myometrial smooth muscle, blocking the action of oxytocin, and inhibition of gap junctions. Progesterone decreases the concentration of myometrial oxytocin receptors which counteracts the effect of estrogens. Progesterone also inhibits PG production by the placenta. In human research, progesterone has been found useful for the maintenance of tocolysis to increase gestational age at delivery or as a preventative agent in women with high-risk pregnancies.[23] Progesterone has been shown to enhance the tocolytic effect of some of the beta sympathomimetics (ritodrine) when used in human patients.[24] Progesterone prolongs gestation length when administered during advanced pregnancy and, as a result, chances of dystocia increase due to additional weight gain of the fetus.

Prostaglandin synthetase inhibitors

Prostaglandins are known to be important mediators in uterine contractility. At the time of parturition, there are increased concentrations of arachidonic acid, PG E_2 and $PGF_{2\alpha}$. Prostaglandins increase intracellular free calcium levels which may increase the frequency of uterine contractions. Thus, nonsteroidal anti-inflammatory drugs that inhibit PG synthesis in the uterus have been considered for use as tocolytics. Indomethacin, a product used in human medicine, has been studied, although with a small sample size, in human patients and was found to delay parturition for 48 hours. Indomethacin is relatively safe as far as the maternal side effects, but crosses the placenta and causes concerns regarding fetal pulmonary hypertension, gastrointestinal inflammation, and hemorrhage.[22] Flunixin meglumine (Banamine®, Intervet/Schering-Plough Animal Health), has been considered for use as a tocolytic agent in cattle based on its ability to block PG synthesis. Although the effects of flunixin meglumine have not been evaluated in a controlled study, the label actually warns against use of flunixin meglumine in late gestation as it is "known to have the potential to delay parturition through a tocolytic effect".[7] One study evaluated the effect of flunixin meglumine on uterine contractility on a small group of postpartum cows and concluded that flunixin meglumine inhibited PG production by more than 80% and decreased spontaneous uterine motility.[25] The effects of flunixin meglumine on uterine contractility have not been evaluated in the pregnant cow.

Calcium channel blockers

Calcium channel blockers have been used for their tocolytic effects in human medicine since the 1980's. Calcium channel blockers work to inhibit uterine contraction by blocking the influx of calcium into the cells of the myometrium through disruption of the voltage-operated calcium channels.[22] Some studies indicate that nifedipine is as effective as magnesium sulfate and beta agonists with fewer side effects. Limited research has shown that the calcium channel blocker, nifedipine, is capable of blocking xylazine-induced uterine contractions in goats. One study found that nifedipine at 80 mcg/kg given intravenously was able to delay parturition in sheep for six to seven hours.[26] Side effects associated with the use of calcium channel blockers for tocolysis include fluid retention and decreased cardiac output which can result in pulmonary edema.[22]

Epinephrine

Epinephrine is an adrenergic that has both α and β activity; therefore, it is capable of relaxing smooth muscle. The use of epinephrine in veterinary medicine has been primarily limited to emergency situations to treat anaphylactic shock or cardiac resuscitation and because of its vasoconstrictive properties as an additive to local anesthetics to decrease absorption and prolong effect.[7] However, epinephrine has also been administered to cattle at 10 cc per cow of the 1:1000 solution as a slow intravenous infusion to cause uterine relaxation and quiescence.[6,20] This use of epinephrine in cattle has been primarily to facilitate obstetrical procedures and conditions such as cesarean section, fetotomy, uterine prolapse, and uterine torsion. Uterine relaxation is almost immediate following intravenous administration. Side effects which may be seen with rapid administration and overdose of epinephrine include severe increases in blood pressure, cardiac arrhythmias, pulmonary edema, and dyspnea.[20]

β sympathomimetics

As previously mentioned stimulation of β_1 receptors leads to increased cardiac automaticity, positive chronotropic and inotropic effects, and elevated free fatty acids while stimulation of β_2 receptors causes relaxation of the uterus, vasodilation, and bronchodilation. Although β sympathomimetic tocolytics may be β_2 selective, they do retain some β_1 activity which accounts for the side effects that may be observed.[22] An ideal β tocolytic would be completely β_2 selective. However, no such drug exists. Therefore, the benefit of uterine relaxation is often accompanied by side effects attributable to β_1 activity. Maternal side effects with β sympathomimetics are associated with cardiovascular complications which include tachycardia, arrhythmias, and ischemia. In humans, the most common complication is pulmonary edema which occurs in approximately 5% of patients.[22]

Clenbuterol and isoxsuprine have been widely used to induce uterine relaxation. Isoxsuprine was the first beta sympathomimetic used for tocolysis. One study demonstrated that isoxsuprine was able to induce tocolysis

within 10 to 15 minutes of administration with a duration of one to 1.5 hours.[27] However, the lack of discrimination between β_1 and β_2 receptors and the resulting tachycardia have limited its use. Clenbuterol is a specific β_2-adrenergic agonist and thus has fewer side effects on extrauterine tissues than does isoxsuprine. Clenbuterol also has the longest duration of action (eight to 10 hours) of any of the β sympathomimetics.[28] Terbutaline is another specific β_2-adrenergic agonist that has been used as a tocolytic in humans. Limited research has demonstrated the tocolytic effects of terbutaline in rats, sheep, and buffalo, and preliminary pharmacokinetic data suggests that it may be a useful tocolytic in cattle.[29] Terbutaline shares many of the side effects associated with other beta sympathomimetics which include tachycardia, cardiac arrhythmias, muscle fasciculations, hypotension, and hyperglycemia of both dam and fetus.[29] Although the beta sympathomimetic tocolytics do have some side effects, these side effects are considered to be a minor concern, particularly when using the more specific β_2-adrenergic agonist. Due to the more predictable efficacy and minor side effects, the beta sympathomimetics are generally considered to the best choice for tocolysis.[20]

However, it is important to remember that none of these drugs are approved for use in food animals in the United States. High doses of clenbuterol have been used as repartitioning agents to promote protein deposition while lowering fat deposition which improved carcass composition. This use of clenbuterol has been associated with acute poisonings in humans who consumed meat from clenbuterol-fed animals. In 1990 in Spain, 135 people had to be hospitalized after consuming tainted veal and liver. In 1994, another 140 people suffered from dizziness, heart palpitations, breathing difficulty, tremors, and headaches. In addition, clenbuterol was banned in the United States, Europe, and Canada in 1997 due to reports of aplastic anemia due to human intoxication with clenbuterol subsequent to its use as a repartitioning agent in cattle.[30] ***The use of clenbuterol in food-producing animals remains illegal in the United States.*** However, abuse of clenbuterol in show cattle in United States has been reported.[30]

Use of tocolytic agents

Tocolytic agents have been used historically to treat or to assist in the treatment of numerous conditions in cattle. These uses include 1) threatened abortion/preterm labor, 2) controlled calving/nocturnal delivery, 3) reduction in neonatal morbidity and mortality associated with dystocia, 4) aid in obstetrical operations such as cesarean section and fetotomy, 5) treatment of uterine prolapse, 6) treatment of uterine torsion, and 7) embryo technologies. Research on the use of tocolytics in ruminants has revealed several factors that may affect tocolysis. The parity of animal may be important to consider as heifers were found to respond faster to clenbuterol and have a longer duration of tocolysis when compared to cows.[31] The amount of cervical dilation and the position of the fetus may also have an affect on tocolyis. In animals where the cervix was fully dilated or fetal feet were found to be passing into cervical area, clenbuterol was only able to delay labor for a maximum of a few hours.[32] Another study evaluated the use of clenbuterol for postponing parturition at various stages in cows. This study found that cows treated during the second stage of labor postponed calving by two hours. In addition, cows treated during the first stage of labor calved 5.2 to 9.7 hours later than control animals.[33] Another study evaluated the use of clenbuterol in beef heifers. This study found that administration of clenbuterol during the first stage of labor (cervical dilation of 5 cm) was able to delay parturition by increasing the length of the first stage of labor with no adverse effects on the fetus or the dam.[28] One report suggests that the use of clenbuterol will reduce neonatal morbidity and mortality in dystocia, will aid in obstetrical operations such as cesarean section and fetotomy. The authors of this study also reported less requirement of epidural anesthesia when clenbuterol was administered versus controls, easier correction of malpresentation and malposition, correction of uterine torsion and uterine prolapse, and no increase in the incidence of retained fetal membranes in bovine dytocias.[34] Another study evaluated the use of clenbuterol in seventeen cows undergoing cesarean section and concluded that the use of clenbuterol in these animals resulted in decreased uterine tone to the uterus which allowed for easier exteriorization and suturing of the uterus.[35,36]

Conflicting reports exist as to the usefulness of tocolytics with regard to embryo technologies. Some have speculated that relaxation of the uterus would improve embryo recovery when used in donor animals and increase pregnancy rates in the recipient animals. However, research and subjective evaluation have not shown any significant improvement in recovery rates of embryos or pregnancy rates in recipients.

Conclusions

It is important to remember that the much of the research on the efficacy and side effects of tocolytic drugs have been conducted in the field of human medicine. There are limited studies in ruminants which are burdened by the lack of critical evaluation and subjective interpretation of the studies' results. Among the β-sympathomimetic agents used in reproduction, only clenbuterol and isoxsuprine have been widely used in clinical management of obstetrical disorders apart from embryo biotechnology with encouraging therapeutic results. The efficacy of these drugs is mostly assessed clinically. However successful the beta sympathomimetics may be as tocolytics, their use is still extra-label and the use of clenbuterol is illegal. Thus more research regarding new tocolytic agents,

efficacy, and the pharmacokinetics of these drugs needs to be studied in detail to ensure wide use with awareness of adverse effects of drug metabolites, if any.

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What's known about selected sperm abnormalities in the bull?

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Abstract

The morphological evaluation of spermatozoa is an important and often overlooked aspect of the bull breeding soundness examination and it is imperative that practitioners not only be able to recognize defective spermatozoa but also to understand the origins of observed defects and provide information related to their effects on the bull's fertility. This review describes the common causes of altered spermatogenesis. It details many of the morphological abnormalities frequently encountered during routine bull breeding soundness exams. Insight is given into their causes as well as the potential impact of each defect on the fertilization process.

Keywords: Bull, spermatozoa, acrosome, proximal droplet; breeding soundness examination

Introduction

The testicle of a bull is a very sensitive organ which is capable of responding to an assortment of insults including heat, hypoxia, radiation, toxicity, and stress as well as the influence of genetic disorders. The response of the testis to these insults often leads to the formation of spermatozoa with observable defects that can be seen during a routine spermiogram. Disturbances in spermatogenesis result in the formation of a wide range of defective spermatozoa which can be observed clinically. Attempts to make both an etiologic diagnosis as well as provide a prognosis for the bull's potential recovery of normal spermatogenesis is one of the goals of a complete breeding soundness examination when excessive numbers of abnormal spermatozoa are observed. This paper will review a select population of sperm defects which could be encountered in veterinary practice and provide their common causes as well as the prognosis for fertility when known.

Causes of altered spermatogenesis

The two most common insults which affect bulls on pasture appear to be heat and stress.[1] The affects of trauma, elevated ambient temperature, fever, excessive deposition of fat in the scrotal neck and scrotal frostbite can all lead to increased heat in the scrotum. The impact of the excessive heat in the scrotum leads to altered spermatogenesis and the eventual appearance of increased numbers of morphologically abnormal spermatozoa in the ejaculate. The temperature of the scrotal contents in normal bulls generally varies between 33.0 and 34.5 °C with a temperature gradient of 6 °C from that of core body temperature.[2] Numerous studies have shown that even very small increases in testicular temperature can have dramatic impact on spermatogenesis leading to the subsequent appearance of abnormal spermatozoa in the ejaculate.[1,3,4] The testicles of bulls appear to function in a nearly hypoxic state which is in part due to the actions of the pampiniform plexus which essentially eliminates the pulse pressure and slows the blood entering the testicle.[2] When scrotal temperatures increase, the metabolic demands of the testicles increase as well, but there is no corresponding increase in testicular blood flow ultimately leading to testicular hypoxia and subsequent alterations in spermatogenesis. [1]

Stress in the form of illness, pain, herd or social interactions, transportation, and weather conditions can be experienced by bulls. There appear to be disturbances in the endocrine pathways associated with normal spermatogenesis in bulls under stress.[1] The affect of stress has been measured by examining the relationship between cortisol, luteinizing hormone (LH) and testosterone. High cortisol levels in bulls have been associated with reduced levels of LH and testosterone when compared to bulls with normal cortisol; the elevated cortisol levels may interfere with normal spermatogenesis.[5]

The toxic effects of gossypol on spermatogenesis are well-recognized and the characterization of the of abnormal spermatozoa has been the subject of several studies.[6,7] The spermatoxic effects of gossypol appear to be related to both the dose and duration of consumption of the phenolic compound produced by the cotton plant.[8] The incidence of gossypol-induced altered spermatogenesis in bulls is likely low due to the rumen's ability to detoxify the gossypol. However, high intake of free gossypol, albeit rare, can overwhelm this system and create toxicosis.[7]

Genetic causes of sperm abnormalities are not as common as environmental causes but are becoming more recognized because of improved diagnostic tools.[9] These defects have been shown to either consistently or occasionally have a genetic mode of transmission. With the ability to scrutinize large numbers of AI sires and their progeny a number of sperm defects have been classified as genetic in nature.

Distal midpiece reflex

The distal midpiece reflex (DMR) is the most common tail abnormality encountered when evaluating the morphology of bull sperm.[10] The typical appearance of a DMR is that of a distinct hairpin bend of the tail at the

location of the distal midpiece, however there can be varying bending patterns noted in the tail giving the affected spermatozoa several different appearances. A consistent finding is the presence of a cytoplasmic droplet noted within the bend of the tail.[10] When this defect is observed in live samples, the spermatozoa will appear to be swimming in a reverse motion often in a circular pattern. [10]

The DMR defect can be induced experimentally when spermatozoa are exposed to hypotonic solutions or when cooled very rapidly.[10] This is critically important when preparing a slide for staining. Many of the morphology stains used today (ie. eosin nigrosin) are hypoosmotic and will create a similar defect if spermatozoa are exposed for an extended time.[10] It is critical that the slide be prepared properly and dried quickly so as to reduce the chance of creating this defect iatrogenically. One striking difference in the defects which occur naturally and those that are caused in vitro is the presence of a cytoplasmic droplet within the reflex of the tail. When a large number of DMR defects are noted without the concurrent presence of a cytoplasmic droplet the possibility of an artificial cause should be investigated.[10]

The DMR defect is produced in the corpus and cauda epididymis where the spermatozoa still have a distal cytoplamic droplet which is present in the bending of the tail.[10] The evidence which supports the epididymis as the origin of this defect is based on appearance of this defect after known testicular insults. Semen evaluation of 606 bulls prior to a severe snowstorm showed the percentage of bulls with greater than 15% DMR to be only 10.9% whereas of the 117 bulls examined 3-4 days after the snowstorm 45.3% had greater than 15% DMR defects.[10] A significant increase in the DMR defect was observed 6-12 days after a brief period of scrotal insulation.[10]

Other recognized causes of this defect include treatment with estrogens, induced hypothyroidism and fever. All of these conditions have been shown to reduce testosterone levels, which appears to adversely affect the epididymal environment leading to the formation of the DMR defect.[10]

The fertility of ejaculates containing high numbers of spermatozoa with DMR defects has not been critically evaluated however it has been observed that the defect could be found in normal fertile bulls with a prevalence up to 25%.[10] Owing to the fact that this defect is of epididymal origin, it would seem that any effect on fertility would be short-lived provided the inciting insult was removed. Since affected spermatozoa would swim in a reverse fashion it is unlikely that they would be able to participate in fertilization thereby could be compensated for by additional spermatozoa. There is no evidence that spermatozoa with the DMR defect are capable of regaining normal function.[10] Semen evaluation in affected bulls should be performed often to detect changes in prevalence of the DMR defect that could help identify an inciting cause.

Knobbed acrosome

The knobbed acrosome defect has been described as a refractile or dark-staining area or eccentric thickening often giving a beaded appearance to the apex of affected spermatozoa.[10] More commonly however, it appears with the apex of the spermatozoa having a flattened or indented acrosome.[10] With the availability of electron microscopy, studies have shown that with both the beaded and indented forms the abnormal acrosome folds back on the sperm apex and a few affected spermatozoa may show a bead-like protrusion from the apex of the spermatozoa. The folding back of the acrosome may also result in a bending back of the apex of the nucleus which causes the apex to appear to have an indentation.[10,11]

The knobbed acrosome defect results from altered spermatogenesis caused by either environmental or genetic factors leading to abnormal development of the fine structure of the plasma membrane making it more susceptible to structural and functional changes.[9,12] It has been shown that spermatozoa with the knobbed acrosome defect lack membrane integrity which can lead to premature capacitation and subsequent acrosome reaction however the exact mechanisms involved remain elusive.[11,12]

It is generally accepted that bulls whose semen contains a high percentage of knobbed acrosome defects will have poor fertility.[10] The exact mechanism by which this defect causes reduced fertility has yet to be elucidated, however it has been theorized that spermatozoa with abnormally shaped heads including the knobbed acrosome defect may have altered motility characteristics which could impair the passage of spermatozoa through the female reproductive tract.[13] Spermatozoa containing the knobbed acrosome defect could also have altered sperm-oocyte binding and zona penetration.[13] In vitro fertilization (IVF) models have shown that knobbed acrosome affected spermatozoa have a reduced ability to bind the zona pellucida and could not penetrate the zona pellucida.[11] Thus the effect of the knobbed acrosome defect on fertility appears to be related to both altered passage as well as impaired plasma membrane function.[12] In vitro studies also indicate there could be compromised fertility associated with apparently normal spermatozoa from males with many knobbed acrosome defects.[14] These normal appearing spermatozoa have been shown to undergo premature capacitation and have spontaneous acrosome reaction as well as evidence of chromatin condensation.[12]

This sperm defect has been associated with infertility in a number of species including bulls, boars, rams and stallions.[11] In cattle this defect was first reported in the Friesian breed in the 1940's however it has since been

seen in Charolais, Simmental, Maine Anjou, Salers, Horned Hereford, Angus and Normande.[9,15] This defect is associated with an autosomal sex-linked mode of transmission in the Friesian breed of cattle and in boars.[9] There appears to be evidence to support a genetic cause of this defect in both the Charolais breed and Angus cattle in North America. [9,15]

Cytoplasmic droplet

The presence of a cytoplasmic droplet is common on a small number of spermatozoa in the ejaculate of fertile bulls. The cytoplasmic droplet is a spherical mass of cytoplasm that is typically found in one of two locations on the spermatozoa. It is considered a proximal droplet when located in the proximal midpiece and a distal droplet when surrounding the midpiece just proximal to the annulus.[10] Droplets are rarely observed in an intermediate location due to the rapid migration of the proximal droplet to the distal location prior to shedding.

Cytoplasmic droplets are formed during spermiogenesis when the spermatid changes from its round shape to an elongated shape and cytoplasm is pulled from the head region towards the tail.[10] During this transition the Sertoli cell molds this cytoplasm into a lobule called the residual body.[10] At the time of spermiation the stalk connecting this residual body to the spermatid is severed and leaving the droplet of cytoplasm in the proximal neck region of the spermatozoa.[10]

As spermatozoa enter the caput epididymis almost 85 percent will have a proximal droplet. As spermatozoa move through the epididymis and maturation occurs the proximal droplet moves to a distal location and is eventually shed. By the time the spermatozoa reach the caudal epididymis over 60 percent of the spermatozoa have a distal droplet.[10] The loss of the cytoplasmic droplet appears to be associated with the gaining of motility in the epididymis.

It is very common to find a high incidence of proximal droplets in bulls that are approaching puberty, however with repeated collections over the following months the percentage of affected spermatozoa generally drops substantially. In yearling bulls a major cause of failure to pass a breeding soundness examination is often the presence of a large percentage of proximal droplets. In data collection from Colorado, 12-26% of yearling bulls failed to pass an initial breeding soundness examination with 6.3% of these failures attributable to proximal droplets.[16] As bulls mature however, the incidence of cytoplasmic droplets tends to drop dramatically. When over 1500 bulls of various ages and breeds were examined the percentage of bulls whose ejaculates contained proximal droplets was 67 percent, with the number of affected spermatozoa averaging only 2.7 percent.[10] In older bulls a high incidence of spermatozoa with proximal droplets points towards abnormal spermiogenesis likely due to a degenerative process of the seminiferous epithelium.[10]

The prognosis for bulls with a high percentage of spermatozoa with proximal droplets varies depending on the underlying cause and the presence of other defects. Recovery has been seen in bulls with profound disturbances of spermatogenesis leading to ejaculates which contain greater than 50 percent of spermatozoa with proximal droplets, however recovery often requires months.[10] In vitro fertilization was used to evaluate the fertilizing potential of semen from young bulls with a high incidence of proximal droplets. It was concluded that fertility was severely compromised but as the bulls matured the incidence of proximal droplets decreased and fertility increased.[17] This study also showed the fertilizing potential of a bull whose semen contained ≥30% spermatozoa with proximal droplets will be low until the incidence of proximal droplets decreases. [17]

Distal droplets are not considered to be a major problem and often indicate insufficient maturation in the epididymis. It has been noted that ejaculates containing a high number of spermatozoa with distal droplets when allowed to incubate for several minutes will almost be totally cleared of the defect.[10]

Crater or diadem defect

The crater or diadem defect is seen in spermatozoa which have a nuclear vacuole or invagination of the nuclear membrane into the nucleoplasm which occurs during spermiogenesis.[18,19] This defect can appear as a "string of pearls" at the acrosome-postacrosomal sheath or as round to elongated white spots which often appear to sparkle.[10] The defect often appears as a surface oriented crater and can range from 1 to over 20 in number.[10] This particular defect is often overlooked with light microscopy on routine eosin-nigrosin stained spermiograms, whereas phase-contrast microscopy and differential interference microscopy allow one to more easily detect this defect. The use of a nucleus stain such as the Feulgen stain should also allow excellent visualization of the nucleus and associated vacuoles.[10]

The incidence of the crater or diadem defect in bulls is generally very high, often approaching 100%; however the percentage of affected spermatozoa within an ejaculate can vary greatly. In a Czechoslovakian study of young and older bulls, nuclear vacuoles were observed in all bulls with the incidence of affected spermatozoa ranging from 3-26%.[10] In one study of bulls at an AI center, all bulls had some defects and 28% of the bulls examined had an incidence of crater defects in excess of 20%.[19]

The precise pathogenesis of this defect has not been elucidated however there are several theories put forth in the literature. Coulter suggested a possible viral etiology based on the presence of viral-like particles within the vacuoles with the thought that the virus may attack the developing spermatozoa.[10] Others have shown that certain insecticides when administered to bulls resulted in an increased incidence of nuclear vacuoles.[10] The most common and accepted theory involves the impact of stress on the process of spermatogenesis. It has clearly been shown that administration of dexamethasone to bulls to mimic a stressful event will significantly reduce their LH and testosterone levels and is thought to impair spermatogenesis and lead to the formation of defective sperm. Studies of the sequential appearance of sperm defects after administration of dexamethasone to bulls clearly show there is an increase in the presence of nuclear vacuoles with the peak incidence occurring around 21 days after dexamethasone treatment.[1] Whether the effects of dexamethasone on spermatogenesis are direct or indirect have yet to be determined. When bulls were studied over long periods of time, fluctuations were seen in the incidence of affected spermatozoa indicating a nonheritable etiology. This is supported by the absence of the defect in significant numbers in the ejaculates of bulls which are sons of a known affected bull.[10] However there is reason to believe that there may be bulls which have a heritable predisposition to produce spermatozoa with the crater defect in response to stress.[10]

It is clear that ejaculates containing high numbers of spermatozoa with nuclear vacuoles are a cause of infertility in the bull. Breeding trials have shown that spermatozoa with multiple nuclear vacuoles have reduced fertilization characteristics both in vivo and in vitro.[18,20] It appears that affected spermatozoa have a reduced capacity to penetrate the zona pellucida as well as reduced ability to form a male pronucleus.[18] With this knowledge, bulls whose semen contains high numbers of spermatozoa with nuclear vacuoles should be monitored with successive semen evaluations as it has been reported that occasionally bulls recover and regain normal fertility.[10]

Dag defect

The Dag defect traces back to a Jersey bull with this name in which this unique defect was first discovered and reported. This heritable defect which occurs during late spermiogenesis is characterized by severe coiling of the tail with fracture of the distal part of the midpiece.[10] A distal cytoplasmic droplet can also be seen associated with this defect.[9] Other features which may be seen include a roughened appearance to the mitochondrial sheath and fracturing of the axonal elements leading to disruption of the mitochondrial arrangement.[10] This defect has been reported as a cause of infertility in several breeds including the Jersey, Polled and Horned Hereford, and the Swedish Red and White. In these particular infertile bulls the incidence of the Dag defect was generally greater than 50%. This defect can be found in the ejaculates of bulls with normal fertility however the incidence of affected spermatozoa rarely exceeds 5%.[10] When the incidence of this defect approaches 50% there appears to be a profound impact on fertility.[10]

A genetic basis for this defect was proven in the Danish Jersey breed through selected breeding of a suspected carrier to 120 of his daughters which produced 6 bulls with the typical defective spermatoazoa.[10] There may be breed specific differences in the exact pathogenesis of this defect because investigators have noted distinct differences in the microanatomy of affected spermatozoa from various breeds.[10]

Pyriform or tapered heads

The pyriform defect is the most common defect of head shape and it appears as a pear-shaped head with a pronounced narrowing of the postacrosomal area.[10] There are many variations of the pyriform defect that range from almost imperceptible to those with severe narrowing all of which can occur in the same ejaculate. The tapered head shape is slightly different than the pyriform head shape in that the entire nucleus is narrow and the head appears elongated.[10] Both of these head defects can be found in the same ejaculate and affected spermatozoa always appear smaller than their normal counterparts. These two defects appear closely related in origin and may be categorized together.[10]

The incidence of the pyriform or tapered head defect occurring in more than 15% of spermatozoa in an evaluation of over 1300 range beef bulls was determined to be 8.7%. This was was lower than the 16.4% incidence found in another study of 216 dairy and beef AI sires.[10] The presence of this defect in low numbers however is a fairly common finding in the ejaculate of many fertile bulls.

The pathogenesis of the pyriform or tapered head defect has not been proven but appears to occur secondary to some disturbance of thermoregulation or an endocrine aberration leading to impaired testicular function.[10] Over-conditioned bulls which have excess fat in their inguinal and scrotal areas commonly have these defects. The appearance of pyriform head defects in experimentally-induced scrotal insulation peaked at 22 days post insulation and at 24 days in dexamethasone-treated bulls suggesting the damaging effects occurred during nucleus condensation and shaping.[1]

The effects of spermatozoa with pyriform or tapered heads on fertility appear to be related to their reduced ability to bind and penetrate the zona pellucida however there does not appear to be an increased incidence of embryo or fetal loss provided fertilization occurs.[10] Work by Saacke investigating the accessory sperm population has also shown that the severity of the head defect may dictate the accessibility of spermatozoa to the ovum thereby limiting the possibility of zona binding.[21,22]

The prognosis for bulls exhibiting large numbers of pyriform or tapered heads in their ejaculates varies depending on the underlying cause. When a cause such as altered testicular thermoregulation can be identified and corrected, the prognosis is generally good provided enough time is allowed to resume normal spermatogenesis. However in bulls which have high numbers of pyriform or tapered heads in which there is no apparent reason for the altered spermatogenesis the prognosis is generally poor for recovery.[10] The percentage of affected spermatozoa can generally be used as a prognostic indicator.

Detatched heads

The presence of detached heads on routine evaluation of a bull's spermiogram is relatively common. This defect is easy to identify during evaluation of a sperm morphology slide. The incidence of this defect in bulls of normal fertility and various ages appears to be around 5 percent.[10] A number of conditions have been associated with the presence of increased numbers of detached heads. In one study of eight bulls with testicular hypoplasia the incidence of detached heads ranged between 39 and 93 percent. However, of these eight bulls, seven were traced back to a common ancestor and the possibility that the high incidence of detached heads was inherited could not be overlooked.[10] The relationship between detached heads and testicular hypoplasia is not consistent among all bulls with this condition. In a study of 141 bulls with testicular hypoplasia the percentage of detached heads was only 6.9 percent.[10]

There appears to be a condition in bulls that mimics the clinical appearance of "plugged ampullae" in stallions. The incidence of this condition was found to be 1.1% when over 1300 bulls were evaluated. The characteristics of the ejaculate consist of a very large volume of highly concentrated semen which contains a high proportion of dead spermatozoa as well as 15-45 percent detached heads.[10] These bulls appeared to improve with frequent repeated collections however the incidence of this defect would be higher after periods of sexual rest. The most common theory is that these bulls have a failure of normal sperm transport with accumulation of spermatozoa within the epididymis and ampullae.[10]

Other conditions that have been shown to cause a transient increase in the number of detached heads include: seminal vesiculitis, epididymitis, or any condition which leads to failure of normal testicular thermoregulation. Lameness which causes the bull to lie down for long periods of time with resultant failure of normal thermoregulation is known to increase the number of detached heads.[10]

Abaxial attachment of the tail

Abaxial attachment of the tail is an infrequently encountered defect of bull spermatozoa. In a retrospective analysis of semen analysis from 1049 range and AI bulls the percentage of bulls found to have the defect was 10.5% and only 0.48% of bulls had semen with greater than 50% abaxial attachment of the head.[23] It is considered a normal finding in the boar and stallion however its significance in the bull remains unclear. There have been reports of sterile bulls that had an increased number of spermatozoa with abaxial attachment of the head however there were no controlled breeding trials performed.[23] In three controlled experiments comparing the fertility of bulls with high numbers of abaxial tail attachement to that of known fertile bulls there was no difference in all fertility parameters measured between bulls.[23] The combined results of these experiments indicate that abaxial attachment of the tail does not impact fertility and should be considered a normal morphological variation of bovine spermatozoa.[23]

Conclusion

There has been a strong correlation between morphologically abnormal sperm and some degree of infertility for many years. It is important to not only be able to recognize morphological defects of spermatozoa but also to provide the client with possible etiologic causes of the defects observed and make suggestions as to their impact on fertility. With the use of routine staining procedures most defects can be observed when examined at 1000X with light microscopy. Electron microscopy may more accurately identify the exact defect present in affected spermatozoa allowing a more in-depth understanding of the sperm abnormalities.

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Nutriceuticals and other drugs used to enhance fertility in stallions

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Abstract

Until recently, there has been little information regarding the ability to improve semen quality or fertility of stallions through alterations in diet. This paper presents evidence from work in humans and other species, including the stallion, which indicates that dietary supplementation with fatty acids, polyamines, vitamins or antioxidants may indeed have the potential of improving semen quality, particularly in those individuals who have marginal semen quality prior to supplementation.

Keywords: Stallion, semen quality, dietary supplementation, fatty acids

Introduction

Over the years, horsemen have been supplementing their animals' diets with various products in an attempt to enhance performance and overall well being. Most of these products have been geared toward improving stamina, hair coat, joint function and hoof growth. Historically, supplements touted to improve the breeding performance of stallions have not proven to be efficacious. Recently however, supplements have become available that show real promise in this regard.

Fatty Acids

Most fatty acids are straight-chain compounds, with an even number of carbon atoms. Chain-lengths can range from two to 80 but most commonly range from 12 up to 24. With a chain length from two to six, they are called short-chain, from eight to 10 they are called medium-chain and 12 up to 24 they are called long-chain fatty acids. Among straight-chain fatty acids, the simplest are referred to as saturated fatty acids. They have no double bonds and cannot be altered by hydrogenation. When double bonds are present, fatty acids are said to be unsaturated. They are called monounsaturated fatty acids (MUFA) if only one double bond is present and polyunsaturated fatty acids (PUFA) when multiple double bonds are present.

The double bonds are counted from the methyl group determining the metabolic family, noted by n-x (with n being the total number of carbon and x the position of the last double bond). For example, linoleic acid is also named 18:2 n-6 in the shorthand nomenclature. Therefore this PUFA has 18 carbon atoms, 2 double bonds and there are 6 carbon atoms from the last double bond to the terminal methyl group. The number following "Omega-" in Omega-3 and Omega-6 fatty acids indicates the position of the first double bond, counting from the terminal methyl group on the molecule. Hence, linoleic acid (18:2 n-6) is an Omega-6 fatty acid. Omega-3 fatty acids cannot be converted to Omega-6 fatty acids or vice-versa.

Omega-3 fatty acids play an important role as structural membrane lipids in all cells. They are also precursors to reactive substances such as prostaglandins and leukotrienes, and possess anti-inflammatory, antiarrhythmic, antithrombotic and vasodilatory properties. There are also data to indicate that dietary supplementation with Omega-3 fatty acids to horses may modify the response to endotoxin by reducing the synthesis of potentially harmful cellular mediators. A plethora of equine dietary supplements containing Omega-3 fatty acids and their precursors are currently being marketed.

Semen from virtually all species examined contains relatively large amounts of lipid. Semen lipids play a major role in motion characteristics, sensitivity to cold shock and fertilizing capacity of sperm. Phospholipids are the major lipid components found in semen and they are largely composed of PUFAs.[1] While spermatozoa from all mammals contain high concentrations of PUFAs,[1,2] the combination and distribution of PUFA in semen varies among species.[3] For example, the distribution of long chain PUFAs in stallion spermatozoa is more similar to boars than that of bulls or roosters.[3] Major differences in the lipid content of bull spermatozoa compared to those of boars and stallions are the relative amounts of 22:5 and 22:6 fatty acids. Spermatozoa of bulls have higher levels of 22:6 fatty acids whereas spermatozoa from stallions and boars have higher levels of 22:5 fatty acids.[3] Bulls and roosters produce spermatozoa that are very resistant to cold shock and freeze well, whereas spermatozoa from boars and stallions have very low tolerance to cold shock and in general, freeze poorly.

In particular, docosahexaenoic acid (DHA; 22:6 n-3, an Omega-3 fatty acid) is the major 22:6 PUFA in semen and docosapentaenoic acid (DPA; 22:5 n-6, an Omega-6 fatty acid) is the major 22:5 PUFA. The majority of seminal PUFAs reside in spermatozoa. In men with poor sperm motility, the level of DHA in seminal plasma as well as the ratio of Omega-3 to Omega-6 fatty acids in their spermatozoa was found to be significantly lower than in men with normal semen quality.[4] Studies in the boar and other species have shown that increasing the ratio of DHA

to DPA in semen increases fertilizing capacity and semen quality.[2,5] Conversely, higher levels of DPA relative to DHA results in reduced fertility.[2] The potential role of DHA in spermatozoal motility and membrane stability is supported by the fact that membranes high in DHA are noted for their flexibility, compressibility, elasticity and deformability.[6]

Animals are unable to synthesize PUFAs from saturated or monounsaturated fatty acids. Therefore, they must acquire them from precursor PUFAs in their diet. Transport of PUFAs from the diet to semen has been shown to occur in a number of species including humans,[7] fowl,[5] boars,[2] and rams.[8] Vegetable oils, such as corn and soybean oil, contain high levels of linoleic acid, the parent compound of DPA. Most proprietary equine rations are therefore, very high in linoleic acid, as well as other Omega-6 series fatty acids and their precursors while the precursors for Omega-3 fatty acids, such as DHA, are very low. A diet of this nature would favor the formation of DPA over DHA since conversion of precursors to DPA and DHA uses the same competitive enzymatic pathway. Since high DPA to DHA ratios in semen have been associated with reduced sperm quality and fertility, typical equine diets with an overwhelming availability of n-6 precursors could have a negative impact on quality of stallion semen and its tolerance to cooling and freezing.

While simply supplementing the stallion's diet with precursors to Omega-3 fatty acids such as cod liver oil or flaxseed oil can increase the overall level of Omega-3 fatty acids in semen, this may not result in the desired effects of improved semen quality. For example, supplementing boar diets with cod liver oil did not improve the freezability of semen.[9] However, when a supplement containing pre-formed DHA and antioxidants was added to boar rations, significant increases in semen quality and fertility were observed compared to boars fed a control diet.[2] Feeding the supplement to boars resulted in a number of benefits including a higher DHA to DPA ratio in semen, as well as an increase in total spermatozoal number, spermatozoal concentration, motility score, percentage of normal spermatozoa, and percentage of viable cells.

Cooling and freezing of spermatozoa can induce cellular injury, which is associated with a disruption of membrane lipids, resulting in damage to mitochondria and loss of integrity of both the plasma and acrosomal membranes. These events are accompanied by a loss of motility, viability and fertilizing capacity of sperm, a phenomenon commonly referred to as "cold shock". Differences in the ability of spermatozoa from various animals to resist cold shock appear to be related to their sperm membrane lipid composition.[3] The lipid composition of spermatozoal membranes not only influences the response of spermatozoa to cooling and freezing, but also plays a major role in the physiologic changes leading to fertilization

Most breed registries have allowed the use of cooled, transported semen, and/or frozen-thawed semen for a several years. The use of shipped semen in the cooled-liquid or frozen state offers many advantages to breeders. Unfortunately, there are many stallions that produce semen that is unable to provide acceptable fertility after undergoing the rigors of cooling and storage, and cryopreservation magnifies this reduction in fertility even further. This problem and the promising results observed in pigs prompted equine researchers to investigate whether the addition of a DHA-enriched dietary supplement could result in improvements in equine semen quality.

Researchers at Texas A&M used eight stallions in a 2 x 2 crossover study to determine if feeding a supplement rich in DHA would improve semen quality.[10] The stallions were randomly assigned to one of two treatment groups (n = 4/group). Within these groups, the stallions were fed either their normal diet (control) or their normal diet top-dressed with 250 grams of a DHA-enriched supplement. The feeding trials lasted for 14 weeks after which a 14-week washout period was imposed, during which only the normal diet was fed. Following the wash out period, the treatment groups were reversed for another 14 week feeding trial. Feeding the supplement resulted in a 3-fold increase in semen DHA levels and a doubling of the ratio of DHA to DPA in the stallions' semen. Spermatozoal motion characteristics in fresh semen were unaffected by feeding the supplement and after 24 h of cooling and semen storage, total and progressive spermatozoal motility also did not differ between treatment groups. However, the spermatozoa from stallions fed the supplement exhibited higher velocity and straighter trajectory than those of stallions being fed the control diet. Beneficial effects were more apparent after 48 hours of cooling and storage, where increases in the percentages of spermatozoa exhibiting total motility, progressive motility and rapid motility, were observed in the semen of stallions being fed the supplement. Total sperm numbers and percentage of spermatozoa with normal morphology were unaffected by treatment. However, when stallions were being fed the supplement, the sperm concentration in their ejaculates was almost double that of when they were fed the control diet. Therefore, it is possible that the observed improvements in semen quality after cooling and storage could be attributed, at least in part, to the reduced exposure of sperm to seminal plasma prior to and during processing.

In a subset of four stallions, whose progressive spermatozoal motility was <40% after 24 hours of cooling and storage when they were fed the control diet, feeding the supplement resulted in improvements in mean progressive spermatozoal motility after both 24 hours and 48 hours of cooled storage. Feeding the supplement resulted in similar improvements in motion characteristics being observed in frozen-thawed semen. Total

spermatozoal motility, progressive spermatozoal motility, and percentage of spermatozoa exhibiting rapid motility were significantly higher in frozen-thawed semen of stallions being fed the supplement.

Despite increasing the level of DHA in semen by feeding the supplement, the level of DPA did not decline. The level of DPA in semen remained higher than that of DHA so that the DHA:DPA ratios were always less than one. Because the stallion's rations were typical equine formulations containing corn and soybean oils, which are the precursors that favor the pathway to DPA, the authors speculate that more dramatic improvements in semen quality may be observed if modifications in the main fat content of the diet are incorporated with the DHA supplement.

The authors concluded that supplementing the diet of highly fertile stallions or those that produce sperm that survive cooling does not appear warranted. However, stallions of marginal fertility and those whose spermatozoa have poor tolerance to cooling and freezing would be horses that might benefit most from being fed the supplement. Optimizing levels of DHA and its precursors by altering the diet of marginally fertile stallions, may improve their semen quality sufficiently enough to make them commercially viable for cooling or freezing.

Similar studies were carried out at the University of Arizona. In those studies, three stallions were fed 550 grams of a DHA-enriched supplement in addition to their normal diet for 90 days.[11] Dietary supplementation resulted in a 3.5-fold increase in semen DHA concentrations. Unlike the study performed at Texas A&M, motion characteristics of spermatozoa in cooled and frozen-thawed semen did not differ between supplemented and non-supplemented stallions. However, in this study dietary n-3 PUFA supplementation did result in a 46% increase in daily sperm output and a 15% increase in normal morphology. The most dramatic improvements were observed for one stallion which had the lowest percentages of morphologically normal and progressively motile spermatozoa prior to supplementation. Recently, workers in Uruguay[12] performed a 2 x 2 crossover study similar to the one performed at Texas A&M[10] with 3 stallions/group and treatment consisting of 30 grams of DHA being fed as a supplement for 80 days. In that study, dietary DHA supplementation improved total sperm numbers, sperm morphology and percentages of live sperm. Progressive motility in semen cooled for 48 hours and frozen-thawed semen improved for some stallions. Consistent with the other studies, the improvements were most noticeable for stallions that initially had poorer semen quality. Improvements in semen quality have also been reported for stallions whose diets were supplemented with rice oil.[13]

Polyamines

Both spermine and spermidine are polyamines found in all cells and thought to be essential for replication, growth, and differentiation.[14] It is believed that spermine and spermidine are produced by the prostate and found in the semen of most mammals. Early investigations documented increased spermatozoal motility *in vitro* when spermine was added to spermatozoa from mice, rats, guinea pigs and rabbits.[12] However, the exact physiologic role of spermine and spermidine in semen remains a matter of debate and their functions may be concentration dependent. Micromolar amounts of spermine in semen appear to enhance the acrosome reaction[15] while milimolar amounts appear to inhibit the acrosome reaction.[15,16]

In rams, ejaculates with spermatozoal motility greater than 85% had approximately two-fold higher spermine and total sperm polyamine content than ejaculates with lower motility. Compared to spermatozoa from lambs the spermatozoa of mature rams had approximately three-fold higher levels of spermidine, spermine, and total polyamines.[17] Lower levels of spermidine are found in the seminal plasma of men with idiopathic asthenozoospermia (poor spermatozoal motility) as well as those with asthenozoospermia associated with diabetes compared to normozoospermic men.[18]

To date, no studies have been published which examined the effects of dietary supplementation of polyamines on stallion semen. However, anecdotal information exists from practitioners using an herbal supplement called "SpermAid", which was originally marketed to increase fertility and libido in stallions, as well as to increase testicular hormone production. The active ingredients in this product are the phytochemicals spermine and spermidine, which are found in radish leaves, radish root, cucumber fruit, and oats. Feeding of the supplement is typically initiated three weeks prior to the breeding season. While significant improvements in spermatozoal motility have not been reported with the use of this product, a number of slow breeding stallions have anecdotally shown dramatic improvements in libido.

Vitamins and antioxidants

Dietary supplementation with antioxidants and vitamins has been shown to have beneficial effect on semen quality. Many of these vitamins exert their beneficial effects through their antioxidant properties. However, as with most supplements examined, conflicting evidence exists regarding their efficacy. The conflicting results can be related to the species of the male subjects in the different studies as well as the doses of individual supplements or combination of supplements investigated.

Vitamins C and E are well known for their antioxidant properties and are those that have been the most extensively examined. In rabbits, dietary supplementation with vitamin C, vitamin E or a combination of vitamins C

and E increased total spermatozoal output, spermatozoal concentration and spermatozoal motility while decreasing dead and abnormal spermatozoa in the ejaculate.[19] Analogous findings have been reported for humans and boars.[19,20] In humans, vitamin C was associated with higher spermatozoal numbers and concentrations in ejaculates, whereas vitamin E appeared to exert its effects by improving spermatozoal motility.[20] Similarly, there was tendency for semen production to be greater for boars supplemented with water soluble vitamins, with the effect being less in boars supplemented with fat soluble vitamins.[21] While the intake of high levels of antioxidant vitamins was associated with better semen quality, moderate intake did not appear to be effective.[20] While other investigators were unable to demonstrate any improvements in conventional semen quality parameters from infertile men,[22,23] supplementation with Vitamins C and E did result in a significant reduction in DNA fragmentation.[23]

Work in the stallion is more limited. In 1978, a German investigator gave 10 stallions of three different breeds an emulsion containing Vitamins A and E for eight weeks.[24] Improvements in ejaculate volume, spermatozoal concentration, spermatozoal morphology and spermatozoal motility, including post-thaw motility were reported. However, the improvements were not universal and appeared to differ among breeds. More recently, Russian workers formulated a complex feed additive that included vitamins A, D and E.[25] They reported improved spermatozoal motility in fresh semen and that spermatozoa remained viable longer after freezing and thawing.

Another antioxidant, showing promise for improving semen quality is L-carnitine (levocarnitine). Along with its antioxidant properties, L-carnitine is essential for mitochondrial energy metabolism. Both L-carnitine and L-acetyl-carnintine are found in high concentrations in the epididymis and both forms are accumulated by spermatozoa.[26] In men with asthenozoospemia, combined treatment with L-carnitine and L-acetyl-carnitine was effective in increasing spermatozoal motility.[24] The most significant improvements were seen in men with the lowest numbers of motile spermatozoa prior to treatment. Feeding L-carnitine to boars resulted in higher semen volumes and spermatozoal concentrations thereby increasing the total number of available spermatozoa in ejaculates for artificial insemination.[27]

Conclusions

It is clear that dietary alterations can have an effect on semen quality and in some cases, fertility. Controlled studies in stallions are few, but those investigating fatty acids, in particular Omega-3 fatty acids such as DHA, have shown real potential. Based on work in other species, further studies involving optimal levels of individual supplements and combinations of supplements which could act synergistically to improve stallion semen quality are needed.

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Antibiotics and other additives for semen extenders to enhance fertility

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Abstract

Semen extender formulations have been evolving for over 70 years and this evolution continues. Although milk was found to be suitable as a major component of equine semen extenders over 60 years ago, preparation of the extenders remained tedious and time consuming in part, due to the need to heat the milk before use. With the publication of the formulation for a nonfat, dried skim milk–glucose (NFDSM-Gluc) in the 1970s, a simple, convenient semen extender became available, prompting a dramatic expansion in the use of equine artificial insemination. This basic formulation of the NFDSM-Gluc extender is still in use today, with various modifications. More recent work has further identified the beneficial components of milk, which has led to more defined extender formulations. This paper briefly reviews the development of equine semen extenders and discusses the effect of including various additives such as antibiotics, cryoprotectants, and antioxidants on equine sperm survival and fertility.

Keywords: Stallion, semen extender, additives

Introduction

The search for an optimum semen extender formulation has been an ongoing quest almost from the inception of artificial insemination. Despite the fact that artificial insemination of mares is reported to have occurred much earlier than in cows and much of the early work in the field was done in the horse, the development of effective extenders for stallion semen was outpaced by the development of bull semen extenders.[1,2] This was due in part to the lack of demand for storage of stallion semen, the belief that stallion sperm lacked "the innate resistant factor necessary for storage" and the restrictive attitude of many breed registries toward artificial insemination.[1,3] While a thorough review of the development of equine semen extenders is beyond the scope of this manuscript, a brief review of early formulations should help set the stage for where we are today.

Over the years, numerous extender formulations have been employed in attempts to improve the survivability of stallion semen. Included in these formulations are various combinations and levels of: egg-yolk, sugars, buffers, citric acid, electrolytes, gelatin, glycerin, honey, milk products and even blood serum and follicular fluid.[1,2,3] The milk products have included mare's milk, sheep and goat milk, cow's milk, cream, skim milk, buttermilk, and nonfat dried milk solids (NFDSM).[1,2,3] One of the first extenders for stallion semen was a glucose-sulphate-peptone formulation,[4] which was later modified to a tartaric-glucose-peptone formulation.[5] Peptone is a water-soluble mixture of amino acids and peptides derived from the partial hydrolysis of protein. Although the source of the peptone used in those early experiments is not stated, it is interesting to note that the peptone that can currently be obtained from Sigma-Aldrich (St. Louis, MO, USA) is derived from the enzymatic digestion of the milk protein, casein.

For many years, equine semen extender formulations were either the same or very similar to those used for bull semen. Results were mixed, sometimes being favorable and oftentimes less than satisfactory. After the discovery that adding egg yolk to a buffer improved the survival of bull sperm[6] and lessened its susceptibility to temperature shock,[7] a number of investigators incorporated egg yolk into equine semen extenders.[8,9,10] In 1949, Buĭko-Rogalevič reported that sperm motility was preserved for 8 to 13 days when stallion semen was diluted in an egg yolk-glucose extender compared to 2.5 days when diluted with glucose and that an 85.5% pregnancy rate was achieved with semen stored for 12 to 42 hours in this egg yolk-glucose extender.[9] In a series of experiments, Kühr obtained similar results with sperm survival increasing from 8.2 hours in undiluted semen to 100.8 hours in 7% glucose and to 290 hours when semen was diluted in 7% glucose + 5% egg yolk.[11] Other extender formulations based on successful bull semen extenders were far less satisfactory for stallion semen.[2]] Pace and Sullivan reported that the fertilizing capacity of equine semen was depressed almost immediately after mixing with hydrogen ion extenders.[12] Investigators from several laboratories found that even though various extender formulations could maintain sperm motility, the fertility of semen diluted in these extenders was poor.[3,11-14] The inferiority of these extenders is best exemplified by the fact that pregnancy rates were higher

when similar numbers of sperm were inseminated using raw semen than with extended semen.[12,14,15] As a result, even up through the mid 1970s, it was recommended by some to use raw semen for equine artificial insemination unless the semen was to be stored or unless antibiotics needed to be added to the semen because the stallion was shedding pathogenic bacteria.[3,14,16] When one examines the composition of the extenders used in many of those studies, it is likely that the glycerol they contained contributed to the poor fertility observed.

Milk-based extenders

Milk was used as an extender for stallion semen as early as the 1940s and boiled mare's milk was reported to yield more favorable results than sheep, goat and even cow's milk.[17] One of the major drawbacks of using fresh milk in semen extenders is the need to heat the milk to 92 to 95 °C for 10 minutes in order to inactivate lactenin, which is toxic to sperm.[18-20] Because of the heating and pasteurization involved in their manufacture, use of reconstituted dried milk products is thought to alleviate the need for heating when used in semen extenders. In the late 1950s and early 1960s, the Chinese established a very successful equine artificial insemination program involving 40 stallions and thousands of mares, using semen diluted in a powdered milk-based extender.[21] In comparative studies, Cheng reported that both maintenance of sperm motility and pregnancy rates were higher using the powdered milk extender when compared to fresh mare's milk or sugar-based (glucose or sucrose) extenders.[21,22] Following up on favorable results with bull semen in the late 1950s, workers at Texas A&M evaluated reconstituted buttermilk with glucose added (BMG) as an equine semen extender.[1] Although fertility was not examined, this BMG extender was found to be far superior to mare's milk, cow's milk and egg yolk-glucose extenders for preserving sperm motility for up to four days. The dried buttermilk was an "extra grade" product prepared by a company in Wisconsin and it may be that limited availability of this product precluded its widespread use in semen extenders. However, non-fat dried skim milk had been readily available for years and once Kenney and co-workers[23] published the recipe for a non-fat dried milk solids-glucose extender (NFDMS-Gluc), this 'Kenney extender' as it is known, revolutionized equine artificial insemination in the western world. With the availability of a convenient, reliable semen extender, the use of artificial insemination in horses increased worldwide and the basic formula for this extender has remained virtually unchanged since its publication in 1975. Kenney-type extenders are available from a number of commercial sources, differing primarily in the type and level of antibiotic(s) added to the basic formulation.

Antibiotics

Inclusion of antibiotics in semen extenders is meant to reduce or eliminate bacterial growth in semen, especially when it is stored, and to help control post breeding endometritis. As with many other extender components, the incorporation of antibiotics was based on satisfactory methods employed with bull semen. However, it was found that the levels of antibiotics commonly used for bull semen extenders were toxic to stallion sperm.[1] Berry and Gazder reported that inclusion of 400 I.U./mL of penicillin and 1 mg/mL of streptomycin in their BMG extender was effective in controlling bacterial growth without adversely affecting sperm motility. The original Kenney extender contained either 1,500 I.U. of crystalline penicillin/mL and 1.5 mg of crystalline streptomycin/mL or 1 mg/mL of reagent grade gentamicin. Antibiotics commonly included alone or in combination in equine semen extenders today are: penicillin, streptomycin, polymixin-B, ticarcillin, timentin, gentamicin, and amikacin. Although less commonly used, ceftiofur[24] and pipericillin,[25] have also been shown to be safe and effective antibiotics to include in equine semen extenders. For some very acidic antibiotics, eg. gentamicin and amikacin, buffers also need to be added to adjust pH and it is important to use reagent grade rather than injectable products because the preservatives in the latter can be toxic to sperm.

While sperm motility and fertility of stored stallion semen can generally be maintained or improved by extenders containing any of the antibiotics listed above, the choice of which antibiotic to include in the extender may be determined based on specific needs or circumstances. For some normal stallions, certain antibiotics appear to be more favorable than others for maintaining sperm motility in stored semen. Certainly, for stallions that are shedding specific pathogens into their semen, the choice of antibiotic to include in the extender should be based on the sensitivity pattern of the offending organism(s).

In the 1980s, Colorado State University entered into a licensing agreement with a commercial company to market a NFDSM-Glu extender. The formulation was essentially the same as the Kenney extender except that 1000 IU/mL of polymixin B sulfate replaced gentamicin sulfate as the antibiotic.[26] For a number of years, this extender (EZ –Mixin® original formula, Animal Reproduction Systems, Chino, CA, USA) was used extensively in the industry for both fresh and cooled-stored equine semen. Later, Colorado workers examined the effects of

different antibiotics on motion characteristics in stored semen.[27] Reagent grade amikacin sulfate, ticarcillin disodium, gentamicin sulfate and polymixin B sulfate were added to a nonfat, dried, skim milk - glucose seminal extender at concentrations of 1000 or 2000 μg or IU/ml. They found that overall the addition of antibiotics to extender did not significantly improve motion characteristics of sperm over control samples but that levels of gentamicin sulfate greater than 1000 μg /ml and polymixin B sulfate equal to or greater than 1000 IU/ml significantly reduced sperm motility. These workers concluded that genatmicin and polymixin B greater than or equal to these levels should be avoided in seminal extenders used for cooled semen. Texas A&M workers performed a similar series of experiments, but also evaluated the control of bacterial growth.[24] Results of this study demonstrated that semen stored in extender containing 1000 IU/mL of polymixin B sulfate resulted in the greatest reduction in sperm motion characteristics and the poorest control of bacterial growth. These workers determined that a NFDMS-Gluc extender containing potassium penicillin G (1000 IU/mL) and amikacin sulfate (1000 μg/mL) yielded the best combination of motility maintenance and control of bacterial growth. Individual stallion effects were also noted.

While not an antibiotic, the inclusion of the sugar mannose into semen extenders has been proposed by Illinois workers as an alternative to antibiotics for reducing post breeding bacterial endometritis.[28] Previous work from this laboratory has indicated that this sterioisomer of glucose was able to reduce the adherence of certain bacteria to endometrial tissue.[29-31] Replacing up to 37 mg/mL of glucose with mannose in NFDSM-Gluc semen extender did not affect the fertilizing capacity of sperm when immediate insemination was performed on reproductively healthy mares.[28] However, whether the inclusion of mannose in semen extenders can control bacterial growth in semen or maintain acceptable pregnancy rates with cooled-transported semen or in susceptible mares requires further study.

Variations on basic components

Texas A&M workers also developed another variation of the Kenney extender. This formulation not only contained the penicillin-amikacin combination but also reduced the level of glucose from 4.9 mg/mL to 2.65 mg/mL with the addition of sucrose at 4.0 mg/mL. This TAMU extender has proven to be an excellent extender for use in fresh, cool-stored breeding programs and also as a base extender for frozen semen after the addition of egg yolk and glycerol.

French workers developed a successful milk-based extender that has been widely used for frozen semen. In addition to sterilized skim milk, glucose and antibiotics, the base INRA 82 extender also contains lactose, raffinose, sodium citrate and potassium citrate to which egg yolk and glycerol are added prior to freezing.[32] More recently, studies which evaluated the effects of different milk fractions on sperm survival resulted in the development of a defined milk protein extender (INRA 96) for use with fresh and cooled semen.[33] In this extender, skim milk is replaced with the specific milk component; native phosphocaseinate (NPPC) in a Hank's salts solution supplemented with HEPES, glucose, lactose (HGLL) and BSA. While no difference was detected in sperm motility after 24 h storage of semen in either INRA 82 or INRA 96, fertility was higher for the semen stored in INRA 96.[33] This extender was also shown to be as efficient at preserving sperm motility and fertility when semen was stored at 15 °C as when stored at 4 °C.[34] This extender can also be used for freezing stallion semen. A fertility trial was conducted comparing INRA 82 and INRA 96 supplemented with egg yolk and glycerol. Although motility parameters were significantly higher in INRA 82 than in INRA 96, the INRA96 extender significantly improved per-cycle pregnancy rates compared with INRA82 (71% versus 40%) in a total of 84 mare cycles.[35]

Japanese workers reported that the addition of 2% casein and 5% egg-yolk to a boar semen extender (Modena) resulted in superior sperm viability in cooled stored semen compared to Kenney extender.[36] Semen stored in this extender at 5 °C resulted in 14 of 22 mares becoming pregnant within 72 h of storage and 3 of 4 mares becoming pregnant with semen stored within 96 to 120 hours. The problem with adding egg yolk to extenders is that it compromises the ability to accurately assess sperm motion characteristics if the extender is not clarified.

Workers in Austria, evaluated another defined milk protein extender (EquiPro®, Minitüb, Tiefenbach, Germany) containing caseinate, selected whey proteins, a range of different sugars and glycine.[37] Interestingly, casein and glycine were components of early extenders such as the CGH-27 extender described by Nishikawa in 1975.[13] After 48 and 72 hours of storage at 5 °C semen stored in EquiPro® extender reportedly had

significantly higher sperm motility than that stored in a Kenney extender. They also reported that centrifugation and removal of 90% of the seminal plasma, which is replaced by the defined milk protein extender, increased the longevity of sperm during storage.

Seminal plasma

The adverse effects of seminal plasma on the survival of equine sperm were recognized as early as the 1930s in the investigations of semen storage.[5,38,39] Many early investigations not only examined various extender formulations, but also optimal dilution ratios of semen in extender. More recently, Colorado workers demonstrated that when using milk-based extenders, complete removal of seminal plasma resulted in significant reductions in the sperm motion characteristics of cooled equine semen whereas suspension of equine sperm in extenders containing 5 to 20% seminal plasma maintained motion characteristics for over 72 hours of cooled storage.[40,41] Subsequently, it has been widely recommended that dilution ratios of at least three to four parts extender to one part semen be used for cooled equine semen, so that the level of seminal plasma does not exceed 20 to 25% by volume and the sperm concentration remains between 25 x 10^6 and 50 x 10^6/mL. For some stallions, whose sperm do not tolerate the rigors of cooling and storage using simple dilution, centrifugation and partial removal of the seminal plasma to achieve even lower levels ($\leq$ 10 to 12%, v:v) may be necessary to optimize sperm survival.[4] However there are other stallions whose seminal plasma is so toxic to their sperm that complete removal is necessary to avoid a rapid reduction in longevity. When complete removal of seminal plasma is required, alternatives to typical milk-based extenders must be employed.

Padilla and Foote[43] demonstrated that after centrifugation and complete removal of seminal plasma, the motility of cooled-stored equine sperm was greatly improved when resuspended in a Kenney's NFDSM-Gluc extender supplemented with a high-potassium modified Tyrode's medium (KMT). However when KMT extender was used in the presence of seminal plasma, motility was reduced, indicating an interaction between seminal plasma and the extender composition. Workers at Texas A&M confirmed these results, and went on further to demonstrate that fertility was maintained with 13 of 17 mares becoming pregnant using semen stored for 48 h in the KMT extender.[44] Other work from this laboratory demonstrated that both motility and DNA integrity were maintained in sperm from which seminal plasma was removed, followed by resuspension in either Kenney extender or modified Kenney Tyrodes-type extender [45]. Other investigators have shown that motion characteristics and acrosomal integrity of sperm are maintained when stored 48 hours after seminal plasma removal and resuspension in a Kenney extender supplemented with commercially available phosphate buffered saline containing glucose and pyruvate.[46] Investigators from this laboratory also reported pregnancy rates of 75% (3/4) and 88% (22/25), when this extender was used with semen from two poor cooling stallions in a commercial cooled-transported semen program.[47]

The effects of seminal plasma are not always deleterious and appear to be stallion dependent. When semen from stallions that exhibited low post-thaw sperm motility (<20%) was supplemented with seminal plasma from stallions that produce semen with high post-thaw motility, greater numbers of spermatozoa survived cryopreservation.[48]

Cryoprotectants

The discovery in the 1930s that the addition of egg yolk to suitable buffers significantly increased the fertilizing capacity of stored sperm from a number of species resulted in the widespread use of artificial insemination in dairy cows.[6,49,50] Most equine freezing extenders consist of milk, egg yolk, glycerol, various sugars, and electrolytes. While chicken eggs are the most common source of yolk used in semen extenders, yolk from other species has been substituted with favorable results. One study demonstrated that sperm motility parameters were improved when stallion semen was frozen in lactose EDTA extender supplemented with duck egg yolk rather than chicken egg yolk .[51]

Glycerol has been one of the most widely used cryoprotectants for frozen semen. However, while a higher level of glycerol often yields better post-thaw sperm motility, higher glycerol levels are also contraceptive in the mare.[12,52,53] Levels of glycerol in early studies ranged from as low a 1% to as high as 10%. In fact, the first reported pregnancy using frozen-thawed epididymal stallion sperm was obtained using an extender containing 10% glycerol (glycerin).[54] Many equine semen freezing extenders currently contain approximately 4% glycerol, but some European studies suggest that a final glycerol concentration of 2 to 3.5% may be most appropriate for

cryopreservation of equine semen.[55,56] However, INRA 96 with 6% glycerol was recently reported to improve survivability of cryopreserved equine sperm while not adversely affecting fertility.[57]

Because of the tremendous variability observed in the post-thaw motility and fertility of stallion semen frozen in conventional extenders, alternative cryoprotectants to glycerol have been investigated. In one study, the presence of glutamine at 50 mM was not sufficient to offset the need to use glycerol.[58] However, it was found that 50 mM glutamine added to a 2.5% glycerol medium significantly improved sperm motility compared to classical freezing medium containing 2.5% glycerol. These workers concluded that glutamine has a synergistic cryoprotective effect with glycerol on cryopreservation of stallion sperm, and suggested that glutamine acts at the extra-cellular level, independently of glycerol.

Recent studies have demonstrated that both methyl formamide and dimethyl formamide could protect stallion sperm from cryodamage as effectively as glycerol, and it was suggested that these cryoprotectants might provide an alternative for stallions that have poor post-thaw sperm motility when frozen in glycerol.[59] A new freezing extender Botu-Crio® (Biotech Botucatu, Botucatu,Sao Paulo, Brazil) has recently been made commercially available. The main difference in Botu-Crio® compared to other freezing extenders, is the combination of glycerol and methyl formamide as the cryoprotectant. Fertility was assessed for good and poor freezing stallions in a retrospective analysis of 355 cycles of mares bred with semen frozen in a glucose–EDTA–lactose extender containing glycerol and on 98 mare cycles for semen frozen in Botu-Crio®.[60] While there was no difference in fertility in the good freezing group between extenders, fertility of the poor freezing group was significantly better for semen frozen in Botu-Crio®. It was concluded that the Botu-Crio® extender appears to improve the post-thaw quality and fertility of stallions with semen that is considered to have poor freezability.

Antioxidants

Oxidative damage to sperm during storage is thought to be a potential cause of the decline in motility and fertility. Endogenous lipase activity in seminal plasma was suggested to be a contributing factor in the adverse effects of seminal plasma on cooled stallion sperm.[61] Numerous antioxidants have been added to semen extenders, with varying results, in an effort to prevent damage to equine sperm by lipid peroxidation.[62-68] As with many other extender additives, much of the work with antioxidants has examined in vitro sperm characteristics rather than fertility.

The addition of taurine to several different extenders was reported to consistently result in better sperm motility after storage than non-taurine containing extenders.[62] Addition of ascorbic acid was found to increase the percentage of membrane intact sperm stored in a skim milk extender compared to controls.[65] In contrast, another study found that the addition of the enzymatic scavenger catalase, or a variety of water-soluble or lipid-soluble antioxidants did not significantly improve the maintenance of sperm motility in semen stored at 5 °C in a NFDSM-Gluc extender.[64] The addition of 2 mM pyruvate to a skim milk extender was beneficial in maintaining sperm motility for semen stored for 48 hours, and based on embryo recovery rates, also tended to improve fertility.[69] Although lactate dehydrogenase activity was found to be correlated with sperm motility,[70] neither pyruvate nor lactate could protect sperm from a H_2O_2 challenge, and it was suggested that beneficial effects exerted by the addition of pyruvate or lactate to semen extenders were probably resulting from them acting as an energy source rather than as antioxidants.[67] Quercetin was recently reported to protect sperm from peroxidation after challenge with xanthine-xanthine-oxidase.[66] These authors also suggested that addition of quercetin to NFDSM-Gluc extender could reduce lipid peroxidation of sperm and thereby prevent premature capacitation of sperm while still allowing the sperm to capacitate and acrosome react after insemination.[68] However, this latter conclusion was drawn from the ability of sperm in quercetin treated semen extender to acrosome react after challenge with A23187, which is not very physiologic, and the authors rightly suggested that fertility trials should be performed to determine the effectiveness of quercetin on sperm storage.

The value of including additional antioxidants to semen extenders has been challenged by results of more recent experiments, which indicate that there is not a substantial increase in lipid peroxidation during semen storage and that peroxidative damage to sperm membranes is not the predominant cause of reduced semen quality.[71,72] Workers from this laboratory report that the inherent antioxidative activity in stallion semen appears to prevent the formation of reactive oxygen species (ROS) and that the simple addition of extender increases this activity further.[71,72] Other workers have also suggested that although equine seminal plasma contains high superoxide dismutase-like activity, sperm themselves have limited glutathione peroxidase and superoxide

dismutase-like activity.[73] They also suggest that the enzymatic antioxidant activity in equine sperm appears to be predominantly derived from seminal plasma adsorbed onto the sperm plasma membrane and that removal of seminal plasma during semen processing may increase oxidative stress in equine sperm. Brazilian investigators reported that lipid oxidation in the seminal plasma appeared to be a general indicator for sperm damage and suggested that both lipid and protein oxidation may aid in the identification of subfertile stallions, but only during the non-breeding season.[74] They also reported that ROS production levels did not appear to result in compromised sperm DNA integrity, which indicated to them that either the measurements were within physiological levels and/or that there is an efficient antioxidant activity in stallion sperm cells.[74]

Conclusions

The NFDSM-Gluc formulation, with slight variations on the basic components ranging from antibiotics to sugars, remains the mainstay of equine semen extenders. Inclusion of a variety of other components such as cryoprotectants and antioxidants has also been attempted, with mixed results. Differences in laboratory techniques and sample populations of stallions likely contributed to this disparity. Use of defined milk proteins has been the most recent major evolutionary step in the development of universally acceptable equine semen extenders and variations on this theme will continue to fuel further research.

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Streptococcus equi subspecies *zooepidemicus* resides deep in the chronically infected endometrium of mares

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Abstract

Bacterial endometritis is considered a leading cause of infertility in the mare. Detection of the causative agent has traditionally relied on the use of the "guarded swab method". Recently, however, attention has been directed towards alternative detection methods after Nielsen[6] found that a high proportion of infertile mares that were negative for *Streptococcus equi* subspecies *zooepidemicus (S. zooepidemicus)* based on culture from swabs (21/84=25%), were positive based on culture from endometrial biopsies (61/84=73 %) (p<0,0001).

Using fluorescence *in situ* hybridization (FISH), bacteria may be visualized within infected tissue, and their spatial distribution can be used to provide information about bacterial pathogenesis. The aim of the present study was therefore to develop a FISH procedure to visualize *S. zooepidemicus* in order to be able to detect the bacteria in the endometrium of experimentally infected mares and in broodmares diagnosed with clinical endometritis.

Using FISH, *S. zooepidemicus* could be visualized in the endometrium in a majority of the infected mares studied. In the young experimentally infected mares, *S. zooepidemicus* was located superficially in the uterus, and could not be visualized 48 h after inoculation. However, in the chronic endometritis cases, *S. zooepidemicus* was found in distinct foci within the endometrium, often just below the luminal epithelia, but also further down, in the stratum compactum, 300 to 500 µm from the endometrial lumen. No *S. zooepidemicus* could be visualized directly on the luminal surface of the endometrium, but in some instances bacteria were present in the endometrial crypts.

Keywords: Endometritis; *Streptococcus equi* subspecies *zooepidemicus*, localization, chronic infections, equine, fluorescence *in situ* hybridization

Introduction

For more than 80 years endometritis has been considered a leading cause of infertility in the mare. [1, 2] *Streptococcus equi* subspecies *zooepidemicus* (*S. zooepidemicus*) has historically been considered as the main pathogen in equine endometritis. [3-6] Forty years ago, Hughes and Loy[7] demonstrated that a population of mares could be described as either susceptible or resistant to endometritis based on their ability to clear an infection following experimental inoculation of *S. zooepidemicus*. Since then, substantial efforts have been devoted to identify factors that determine whether a mare is susceptible or resistant to endometritis. Compromised uterine clearance and myometrial contractility were demonstrated in susceptible mares[8, 9] and have been identified as important components of the pathophysiology of endometritis. [10-12]

The diagnosis of endometritis in the mare has routinely relied on using a guarded swab followed by bacterial culture and cytological evaluation of exfoliated endometrial material.[13,14] Recently, attention has been directed towards alternative detection methods, after it was shown by Nielsen[6] that the swabbing method was inadequate for detection of uterine pathogens, especially *S. zooepidemicus*. *S. zooepidemicus* were isolated from sixty-three of 84 culture positive mares (75%) based on endometrial biopsy whereas only 23 of the same 84 mares (27%) cultured *S. zooepidemicus* using the swabbing method (p<0,0001).

The higher proportion of positive mares using endometrial biopsy as compared to swabs, indicates that *S. zooepidemicus* may reside below the epithelial lining, in the deeper layers of the endometrium, and is therefore less likely to be detected by the non-invasive swabbing method. Information regarding the localization of *S. zooepidemicus* within the endometrium will affect our perception of *S. zooepidemicus* induced endometritis and potentially affect the choice of treatment regimen. Consequently, we wanted to investigate where in the endometrium *S. zooepidemicus* was located in mares suffering from endometritis.

Together with immunohistochemistry and polymerase chain reaction (PCR), fluorescence *in situ* hybridization (FISH) offers an alternative to traditional detection methods based on bacterial culture.[15] The FISH technique relies on the use of hybridization between bacterial 16S ribosomal-RNA and fluorescent labeled complementary oligonucleotides. After hybridization, detection of individual bacterial cells can be made by fluorescence microscopy.[16] Using FISH, bacteria can be identified in their natural environment, and since spatial distribution within the infected tissue can be evaluated, information regarding bacterial pathogenesis can be generated.[17]

Compared to standard epifluorescence microscopy, confocal laser scanning microscopy (CLSM) has the advantage of a pinhole to exclude fluorescence that originates outside the focal point of the objective lens, thus increasing the resolution of fine structures.[18] As 3D data can be acquired using CLSM, detailed visualization of small structures like *S. zooepidemicus* (0,5-2 µm) within the tissue of interest can be obtained.

The primary aim of this work was therefore to develop a FISH procedure that allowed visualization of *S. zooepidemicus* in the endometrium of the mare, and to demonstrate the localization of the bacteria in the endometrium of experimentally infected mares as well as in broodmares diagnosed with clinical endometritis.

Materials and Methods

Endometrial biopsies were recovered from 4 mares experimentally infected with *S. zooepidemicus* and from 6 broodmares with symptoms of clinical endometritis. Except otherwise stated, all plastic wares were purchased from Nunc (VWR International, Albertslund, Denmark), and all reagents were from Sigma-Aldrich (Vallensbæk, Denmark). The media were prepared with freshly produced Milli-Q water (Millipore, Hedehusene, Denmark).

Endometrial biopsies from mares experimentally infected with *S. zooepidemicus*

Four reproductively normal Standardbred mares aged five to ten years and belonging to the teaching herd at the Faculty of Life Science, Department of Animal Science, were used for the experimental infections. The experimental mares were evaluated for cyclicity and clinical signs of endometritis (transrectal palpation/ultrasound and speculum examination of the vagina) every two to three days for a minimum of 14 days before bacterial inoculation. When mares were in estrus, they were inoculated with a *S. zooepidemicus* isolate cultured from a mare with clinical endometritis (10^9 colony forming units in 10 ml of brain-heart infusion broth). Endometrial biopsies were sampled from the left and right uterine horn before inoculation (0 h) and again 3h, 6h, 24h, 48h and 96h after inoculation.

Biopsies were collected as described by Nielsen.[6] In brief, the mare was placed in a set of stocks, the tail was wrapped, the perineal region washed, and a sterile biopsy speculum was introduced through the vagina into the cervical canal. Biopsies were recovered using an alligator forceps directed to the base of the left and right uterine horn. The biopsy instrument was not guided by rectal palpation. Following collection of a biopsy, a sterile pair of pincers was used to smear the biopsy onto blood agar (5% fetal calf blood, toxin free). Blood agar plates were incubated at 37 °C for 48 h and colonies of *S. zooepidemicus* were identified by the β-hemolysis, colony morphology (mucoid and non-pigmented) and Gram stain.

Experimental mares were re-evaluated by transrectal palpation and ultrasography, a speculum examination of the vagina, and a bacterial culture from endometrial biopsy 18 to 21 days after inoculation.

Endometrial biopsies from broodmares in clinical practice infected with *S. zooepidemicus*

Endometrial samples were collected from broodmares if clinical signs of endometritis were detected, e.g. >2 cm free fluid in the uterus during estrus before and/or after breeding, mucupurulent vaginal discharge, or repeated breeding without establishment of a pregnancy. Endometrial biopsies were collected and bacterial culture performed as described by Nielsen.[6] No information was available concerning the mares or the breeding management including pregnancy status. To increase the chance of visualizing *S. zooepidemicus* only endometrial biopsies from which β-haemolytic streptococci had been cultured were evaluated by FISH. Five biopsies were evaluated.

Fluorescence *in situ* hybridization (FISH)

Oligonucleotide probes. The *Streptococcus* specific oligonucleotide probe, Strept (5`Cy3-CACTCTCCCCTTCTGCAC-3`), used to target 16S rRNA from *S. zooepidemicus* in this experiment was developed by Trebesius et al.[19] As a positive control, the general bacteria probe Eub338 (5`Cy5-GCTGCCTCCCGTAGGAGT-3`) was used. [20] As a negative control, the reverse and complementary Strept probe called Non-strept (5`Cy3-GTGCAGAAGGGGAGAGTG-3`) was used. All probes were purchased from TAG Copenhagen, Denmark.

In situ hybridization of bacterial cells. Prior to hybridization, fixed bacterial cells were bound to poly-L-lysine (Sigma, St. Louis, USA). Teflon coated slides (Novakemi AB, Enskede, Sweden) and dehydrated by sequential washes in 70 and 96% ethanol (3 min. each). Ten µL of hybridization buffer (40% formamide, 20 mM Tris, pH 7.0, 0.9 M NaCl, 0.1% SDS) and 5 ng of probe were applied to each slide followed by hybridization in a humidified moist chamber at 46 °C. The duration of the hybridization time was at least 1 h. Slides were washed in hybridization buffer (46 °C) for 10 m and subsequently transferred to a washing buffer (46 °C) for 15 m. The slides were finally rinsed in MilliQ-water (46 °C) and air-dried in the dark. The hybridization signal intensity was evaluated with the probes Strept, Non-strept, and EUB338.

Preparation of tissue sections and in situ hybridization. After smearing onto blood agar, the biopsies were transferred into 10% phosphate-buffered formaldehyde for fixation, and the following day transferred to 70% ethanol and placed at 5 °C until slide processing. The biopsies were embedded in paraffin, cut into 5 µm thick sections, which were mounted on adhesive SuperFrost/plus slides (Menzel-Gläser, Braunschweig, Germany). From each biopsy 10 tissue sections were made, of which four would be hybridized

with the Strept and four with the Eub probe respectively, while the remaining two slides would be labeled with the Non-strept probe.

Prior to hybridization, the slides were deparaffinized in xylene for 3 m and allowed to air dry. The tissues samples were then dehydrated by sequential washing in 70% and 90% ethanol (3 min each), and allowed to air dry. Lysozyme (5mg/ml in 100mM Tris (pH 8.0), 50 mM EDTA,) was then added and incubated for 20 min at 37 °C and finally washed in Mili-Q water. Hybridization was performed using 100 µl hybridization buffer (40% formamide; 20 mM Tris-HCL, pH=7.0; 0.9 M NaCl; 0.1% sodium dedocyl sulphate [SDS]) containing a total of 200 ng of probe per tissue section. Only one probe was used for each tissue section. Hybridization was conducted in a CMT-hybridization chamber (Corning Inc., Corning, NY, USA) at 46 °C overnight. The slides were then washed in prewarmed hybridization buffer at 46 °C for 15 m and transferred to a washing buffer containing 20% (vol/vol) formamide for 10 m at 46 °C. Finally the slides were washed in Mili-Q water, allowed to air dry before being mounted with a coverslip for microscopy.

Confocal Laser Scanning Microscopy

Imaging was performed on a Leica Confocal microscope (Leica TCS SP2; Leica Microsystems, Bensheim, Germany). The objective used were Leica Microsystems HC PL APO ×10/0.40 or HCX PL APO ×63/1.32 (oil).

To allow visualization of the endometrial tissue, autofluorescence was excited by the 458 nm laser and the emitted light was detected at the interval from 460 to 529 nm, whereas Cy3 and Cy5 were excited by the 543 nm and 633 nm lasers, respectively, and emitted light detected at the interval from 540 to 600 nm and 650 to 750 nm, respectively. Either single images or a stack of images were used to construct three dimensional visualization of the tissue. Image visualization and three-dimensional analysis were performed with the Leica TCS SP2 software.

Results

Verification of probe specificity

FISH on pure cultures of the *S. zooepidemicus* strain used for experimental infections demonstrated that the bacteria were labeled following probing with the Strept- and EUB338- probe, respectively. Bacterial morphology and number could be determined with both probes, whereas no signal was obtained when the Non- strept probe was applied.

Endometrial biopsies from mares experimentally infected with *S. zooepidemicus*

Results following experimental infections with *S. zooepidemicus* including clinical signs, bacterial culture, and FISH are outlined in Table 1.

Table 1: Clinical signs, results of bacterial culture and visualization of *S. zooepidemicus* by FISH at fixed time intervals following uterine inoculation with *S. zooepidemicus* (0 to 96 h).

Time from inoculation (h)	Clinical signs	Bacterial culture	FISH
0	No abnormalities noted	No growth from three mares. Two *S. zooepidemicus* colonies isolated from one mare.	No bacteria visualized

3 and 6	Increasing amount of fluid (echogenic on transrectal ultrasound evaluation) in the uterus. No discharge or small amount of discharge noted on the speculum examination.	Large (>40) number of *S. zooepidemicus* isolated from biopsies from each mare	Large number of bacteria visualized in the uterine lumen and/or aligned close to the luminal epithelium. Bacteria visualized in all biopsies recovered.
24	Echogenic fluid in the uterine lumen, mucupurulent exudates from the cervix	Large number of *S. zooepidemicus* isolated	*S. zooepidemicus* organized in clusters in distinct foci on the luminal epithelium. Very few if any bacteria visualized in the uterine lumen.
48	Echogenic fluid in the uterine lumen, mucupurulent exudates from the cervix – reduced amounts in three of the four mares compared to 24 h.	10 to 20 colonies of *S. zooepidemicus* isolated from all mares.	Few and small clusters of *S. zooepidemicus* visualized on the luminal epithelium. Bacteria visualized in biopsies from two of the four mares.

96	Echogenic fluid in the uterine lumen identified in one of the four mares. Vaginal discharge noted from one of the four mares.	None or max 3 *S. zooepidemicus* colonies could be identified from three of the mares. A total of 14 *S. zooepidemicus* colonies were isolated from one mare (mare with clinical symptoms, left + right horn).	No bacteria visualized in biopsies from the four mares.

Mares experimentally infected with *S. zooepidemicus* presented clinical signs as expected (Table 1). At 24 h after inoculation mucupurulent discharge originating from the uterus was noted in all four mares. The amount of fluid in the uterus and vaginal discharge had decreased by 48 h in three of the four mares. By transrectal ultrasound, a medium amount (2 cm) of slightly echogenic intrauterine fluid could be identified in one of the four mares at 96 h after inoculation, and *S. zooepidemicus* was cultured from the biopsies from this mare. Three of the mares cleared the infection within 96 h, whereas one mare did not. *S. zooepidemicus* was cultured from this mare when she returned to estrus 19 d after inoculation. The mare was then treated on three consecutive days with penicillin (5 million IU), uterine lavage (1-3 L of Ringer's lactate) and oxytocin (10 IU, IM q. 8 h, Intervet®, Intervet Denmark AS). Bacterial culture from a uterine biopsy performed 2 d after the last treatment was negative.

Autofluorescence from the endometrial tissue, following excitation from the 458 nm laser, was adequate to outline the endometrial structures including luminal and glandular epithelium as well as uterine glands and vessels within the stratum compactum. Differentiation of cells in general, e.g. identification of inflammatory cells, was not possible with the described techniques.

In most situations, a standard epifluorescence microscope equipped with the correct filters is adequate to evaluate tissue processed for FISH, and preferred over CLSM because of price and ease of use. Endometrial tissue autofluorescence was helpful in determining endometrial architecture, but autofluorescence prevented discrimination between artifacts and probe signals when a standard epifluorescence microscope was used, even though specific filters were applied. This problem was overcome when CLSM was used.

Using the described FISH protocol, *S. zooepidemicus* could be visualized in tissue sections originating from endometrial biopsies. When biopsies were collected 3 to 6 h after inoculation, *S. zooepidemicus* was localized in large numbers in the uterine lumen and /or in close association with the luminal epithelium. The number of bacteria decreased substantially, especially in the lumen of the uterus when biopsies were collected 24 h after inoculation. At this time, *S. zooepidemicus* appeared in small clusters on the luminal epithelium. Due to the high resolution of the confocal microscope, the characteristic round shape of the *S. zooepidemicus* could be discerned. In biopsies collected 48 h after inoculation, few and very small clusters of *S. zooepidemicus* could be visualized on the luminal epithelia, but only in biopsies from two of the four mares, even though bacteria were demonstrated upon culture from all recovered biopsies. No *S. zooepidemicus* could be visualized in biopsies recovered 96 h after inoculation, although *S. zooepidemicus* was cultured from one of the four mares.

Endometrial biopsies from broodmares in clinical practice infected with *S. zooepidemicus*

Biopsies collected from five broodmares from a clinical practice from which *S. zooepidemicus* had been isolated were processed as described above. Using FISH, *S. zooepidemicus* could be visualized in the endometrium from three of the five mares. In these likely chronic endometritis cases, *S. zooepidemicus* were localized primarily in distinct foci just below the luminal epithelium, but also deeper in the stratum compactum 300 to 500 µm from the endometrial lumen. No *S. zooepidemicus* could be visualized directly on the luminal surface of the luminal endometrium, but in some instances bacteria were present in the endometrial crypts. Tissue remodeling with increased amounts of fibrotic tissue was, in some instances, observed in areas where bacteria were visualized; but in most cases presence of bacteria did not seem to entail changes in tissue morphology.

Examination of endometrial tissue labeled with the EUB338 probe (positive control) revealed a similar bacteria localization pattern as observed using the Strept probe. Control hybridizations without any probe added (negative control) did not result in any specific hybridization signal.

Discussion

Using FISH, we were able to show a rapid decline in the number of *S. zooepidemicus* during the first four days following experimental infection of young mares in estrus. However, in endometrial samples from clinical cases, presumably representing a more chronic infection, *S. zooepidemicus* was demonstrated to reside deeply within the endometrium. To our knowledge this has not been demonstrated before.

In our initial visualization experiments it was possible to visualize *S. zooepidemicus* in biopsies recovered 3 and 6 hours after inoculation, but not in biopsies recovered 24 hours after inoculation, despite the presence of a large number of bacteria in cultures of smears from the same biopsies. It is known that the success of FISH is dependent on optimization of tissue fixation and permeabilization to allow the probe to access the bacteria.[17, 21] In an attempt to increase the permeation of the 16S rRNA probes, the tissue sections were treated with either lysozyme or protein kinase K .[21] A pilot study detected no difference between samples treated with either of the two permeabilizing agents. Increased permeabilization made it possible to visualize *S. zooepidemicus* in the endometrium, independent of time from inoculation. Why permeabilization was necessary to allow visualization in samples recovered 24 h after inoculation, but not after 3 or 6 h, is difficult to determine. The explanation could be the presence of a bacterial capsule 24 h after inoculation, since *S. zooepidemicus* capsule synthesis is usually lost following primary *in vitro* culture. [22]

A high degree of autofluorescence originated from the endometrial tissue, especially using epifluorescence microscopy when excitation was induced with a mercury lamp. This was particularly true when dilated uterine glands were present in the endometrium, likely due to mineralized deposits within these dilated glands [23] and perhaps autofluorescence originating from leucocytes, as shown in humans. [24] Autofluorescence was reduced when CLSM was employed, probably because excitation was induced with only one specific wavelength at a time.[18] The reduced autofluorescence, combined with the capacity to freely select emission wavelength, whereby probe signal could be differentiated from background, made specific visualization of *S. zooepidemicus* in the endometrium possible.

Localization of *S. zooepidemicus* deep in the endometrium of chronically infected mares explains why Nielsen[6] found a higher number of mares were diagnosed with endometritis by culture from a biopsy than from a swab. A recent study using low volume lavage to diagnose endometritis indicated that *E. coli* in some situations is more frequently isolated (42.2%) than β-hemolytic *Streptococcus* (37.6%). [25] Other studies using a swab to diagnose endometritis, also conducted in Kentucky, found β-hemolytic streptococci to be the pathogen most commonly isolated.[5] The difference with respect to pathogens isolated clearly relates to the diagnostic method used and warrants further investigation. The FISH technique might be useful in the future to illuminate differences in the pathogenesis of *E. coli* and *S. zooepidemicus* induced endometritis in the mare.

Recently, restriction fragment length polymorphism (RFLP) was used to identify the genetic profile of *S. zooepidemicus* clones present in mares with endometritis.[26] Genetic profile or RFLP pattern were evaluated in 12 mares before and after treatment with antibiotics. Following treatment RFLP patterns were identical to those before treatment in 11 of the 12 mares, indicating treatment failure. The authors concluded that *S. zooepidemicus* persisted in the endometrium despite treatment. Different systemic or intrauterine treatments are likely to affect streptococci residing in the deeper layers of the endometrium differently. As described by Schlafer, [27] the use of molecular based techniques such as RFLP, amplified fragment length polymorphism,[28] PCR, FISH, etc. are likely to impact our knowledge of the pathogenesis of endometritis and facilitate optimal treatment protocols in the years to come.

The impact of *S. zooepidemicus* residing deep in the endometrium of chronically infected mares on endometrial function is difficult to predict. Recently, it has been suggested that persistent active inflammation in the endometrium can lead to increased mucus production. [29] Riddle, et al.[5] demonstrated the lowest pregnancy rate in mares with the highest degree of endometrial inflammation and correlated a high level of inflammation to isolation of β-hemolytic streptococci. Chronic infections with *S. zooepidemicus* deep in the endometrium might induce inflammation and increase mucus production potentially reducing pregnancy rates. We foresee that by using the approach described here we will eventually arrive at a better understanding of *Streptococcus*- induced endometritis.

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Antibiotics in mare reproduction

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Abstract

Antibiotics are used to treat a variety of reproductive tract infections in the mare. The results of an on-line survey of veterinarians concerning the use of antibiotics in mare reproduction are presented. Listservs were used to acquire the data. Most veterinarians follow the recommendations in the literature for treating mares with reproductive tract infections. However, some veterinarians may mix antibiotics inappropriately, treat mares too soon after breeding, use inappropriate mixed lavage solutions, or use antibiotics in cases that do not warrant their use.

Keywords: Mare, antibiotic, reproduction, uterine, systemic, fungal

Introduction

Antibiotics are used in the mare to treat potential or realized reproductive tract infections including vaginitis, cervicitis, endometritis, metritis, pyometra, and placentitis. Alternatively, infections may be associated with or classified as sexually transmitted diseases, post-mating induced endometritis, acute or chronic endometritis, abortion related, and/or bacterial and fungal infections.[1] Antibiotics are administered either through intravenous/intramuscular routes or directly into the reproductive tract lumen. Antibiotics are naturally occurring or synthetic substances that inhibit the growth of or kill microorganisms. The definition may be limited to substances affecting bacteria or may also include fungi and protozoa. In this paper antifungals will be considered part of the antibiotic class.

There are a few reviews of the use of antibiotics in mare reproduction.[2-4] The choice of antibiotic should be based on culture and sensitivity patterns when possible or based on the most likely organism when not possible. The most common bacteria isolated from the mare's reproductive tract are *Streptococcus equi* subspecies *zooepidemicus* (Gram positive), *Escherichia coli* (Gram negative), *Klebsiella pneumonia* (Gram negative), *Pseudomonas aeruginosa* (Gram negative), *Staphylococcus aureus* (Gram positive), and *Bacteroides* (Gram negative, anaerobe).[5-10] *Streptococcus equi* subspecies *zooepidemicus* and *Escherichia coli* are the number one and two isolates in almost all reports. The most common fungi isolated from the mare's reproductive tract are *Candida* spp and *Aspergillus* spp.[11]

There are many factors that may affect antibiotic effectiveness/clearance such as overwhelming microorganism numbers, presence of uterine fluid/debris, lack of uterine contractility, use of ecbolics, normality of uterine mucocilliary clearance mechanisms, cervical dilation, and dependency of the uterine horns.[12-15] Disruptions of natural barriers to infection, such as previous cervical trauma/scarring, vestibulovaginal fold incompetence (windsucker), and poor vulvar conformation, may also contribute to continued bacterial/fungal contamination.[16]

Intrauterine antibiotic therapy appears to have decreased in use, most likely due to concerns about inducing secondary fungal infections and/or antibiotic resistance and due to new information on the effectiveness of uterine lavage and the use of ecbolics, such as oxytocin and prostaglandin. Antibiotic therapies are now more targeted at specific organisms, are used with more specific disease processes or are used in conjunction with methods to disrupt biofilms or after decreasing bacterial numbers with lavage techniques. This manuscript will describe the results of an on-line survey of veterinarians concerning antibiotic use in mare reproduction and correlate the results to the literature.

Survey on antibiotic usage

Two surveys were conducted with regards to the use of antibiotics in equine reproduction. The surveys were initiated to see what is commonly used in practice vs. what is recommended in the literature. Both surveys were sent to the Equine Clinicians Network listserv (ecn@listserv.vetmed.wsu.edu), the American Association of Equine Practitioner's listserv (aaep_discussion@list.aaep.org), the Equine Reproduction listserv (eqrepro-l@po.missouri.edu) and the American College of Theriogenologists listserv (ACTList@lists.theriogenology.org). The first was initiated in September 2008 (190 respondents) and the second was initiated in March 2009 (109 respondents). The second survey was performed to augment the first survey results. Sixty-one percent of participants in the second survey partook in the first survey.

Approximately 69% of survey participants stated that the primary way that mares are bred in their practice is by the use of fresh cooled semen, 27% are bred primarily by natural cover, 3% primarily with frozen semen and 2% did not provide an answer. The number of years in practice was: <5 years – 7%, 5 to 10 years – 20%, 11 to 15 years – 13%, 16 to 20 years – 18%, >20 years – 39%, no answer – 2%. The percentage of their practice that was devoted to equine reproduction was: <10% - 11%, 10 to 25% - 21%, 25 to 50% - 18%, 50 to 75% - 15%, >75% - 34%, no answer – 1%. The larger number of practitioners in the >75% category most likely reflects the distribution

of the survey to two predominately reproductively oriented listservs (ACT and EqRepro). Veterinarians from 14 countries participated in the survey, with 70% of respondents practicing in the United States.

When asked which bacterial and fungal organisms they encountered most frequently, the overwhelming answers for bacterial isolates were *Streptococcus zooepidemicus* followed by *Escherichia coli*. One practice stated that they had 80% beta-hemolytic Streptococcus isolated from 1400 uterine cultures in their clinic. An antibiotic with both Gram positive and Gram negative properties may be appropriate for the treatment of uterine infections in those cases without culture. Fungal cultures, according to survey results, yielded primarily *Candida* spp followed secondarily by *Aspergillus* spp.

Intrauterine usage of antibiotics.

Antibiotics may be placed into the uterus prior to or after breeding or in association with treatment of suspected or known uterine infections. Dosages for antibiotics commonly used for intrauterine infusion are presented in Table 1. Practitioners responding to the survey stated that antibiotics administered prior to breeding were used for mares that were known to be problem breeders, mares that were repeat breeders, mares with uterine fluid pre-breeding, mares with excessive uterine edema, mares suspected of having an infection (awaiting culture/cytology results), mares suspected of having an infection (owners decline culture/cytology) or strictly at the owner's request.

Survey participants stated that they used post-breeding antibiotics in situations where they knew the mare had previous problems, in mares with uterine fluid, in those mares susceptible to post-mating induced endometritis, in mares bred late in the breeding season, in mares with previous pregnancy loss or as a routine procedure with a single dose of antibiotics, especially in natural cover situations. A study by Pycock found that pregnancy rates were better after a single dose of antibiotics (± oxytocin) post-breeding, especially in older mares (>12 years) and mares mated at the first estrus post-partum.[17] Some Thoroughbred farms may routinely use a single post-breeding antibiotic to limit bacterial contamination from natural cover.[18]

When asked how many days mares were commonly treated with intrauterine antibiotics, the responses were: one day (12%), two days (7%), three days (50%), four days (5%), five days (7%), one week (1%), other (13%), and no answer (5%). Those answering "other" may treat for 1 to 3 days, 3 to 5 days, number of days would depend on bacteria isolated, number of days would depend on presence of fluid, or would never treat a mare with intrauterine antibiotics. It has been recommended, based on endometrial biopsy, that treatment for mild intrauterine infections be performed for 3 days, moderate infections for 5 days, and severe infections for 7 days.[12] The determination of how mares fit into these categories may not be clear in practice and would need to be subjectively based on clinical signs and possible cytologic examination as biopsy results may not be returned for a number of days. It has also been suggested that mares not be treated for more than 2 or 3 days post-ovulation so as to decrease possible negative effects on corpus luteum progesterone secretion from prostaglandin released in response to endometrial irritation caused by the antibiotic or vehicle.[12] Antibiotics should also not be used immediately pre-breeding as high concentrations of antibiotics may negatively affect sperm function.[12]

Forty-three percent of practitioners would increase the volume of antibiotic solution infused to between 50 to 100 ml prior to infusion. Eight percent used the antibiotic without dilution; 19% added extra volume, but kept the total less than 50 ml; 11% added extra liquid so that the final volume was >100 ml; 7% added the antibiotic to the lavage solution; 6% did not answer the question; and 8% provided an alternative answer of "other" which included leaving some lavage solution in the uterus and adding the antibiotic to that fluid, using a 250 ml bottle of fluid for infusion with antibiotics added, or using a 10 ml or 20 ml total volume. The literature has suggested intrauterine infusion volumes ranging from 30 to 200 ml to achieve distribution throughout the uterine lumen.[2] Six grams of ticarcillin, for instance, has a higher intrauterine concentration over time when a 250 ml volume is infused rather than a 60 ml volume.[19] With large volumes, however, reflux of fluid back through the cervix could occur, diminishing the overall dose. A more appropriate recommendation may be to maximize the volume of an antibiotic solution while considering the relative size and position of the uterus. Multiparous mares would naturally require a larger volume, while nulliparous mares should require less. With a dependant uterus, infused fluids tend to pool in the base of the uterine horns making it difficult to achieve uniform coverage of the endometrium; consequently, systemic antimicrobials may be a good choice in these mares.

The most common antibiotic used for intrauterine treatment prior to receiving culture/antibiotic sensitivity results by veterinarians who participated in the survey was ceftiofur (21%), followed by gentamicin (19%), ticarcillin with clavulanic acid (13%), ampicillin (12%), other (12%), procaine penicillin (5%), amikacin (5%), potassium penicillin (3%), and ticarcillin (3%). Nine percent of survey participants did not answer this question. The category "other" included combination of penicillin and gentamicin (2%), penicillin and neomycin (2%), ampicillin and gentamicin (1%), oxytetracycline, framomycin, framycetin, cefquinome, cefazolin, or chloramphenicol. Interestingly, procaine penicillin was used even though there are no dosages reported in most

published reviews. Some practitioners had concern about residues that may be left within the uterus with the use of the procaine penicillin suspension. Enrofloxacin has been administered by intrauterine infusion without causing more than a moderate inflammatory response,[20] but there are other reports that the basic pH of enrofloxacin is very irritating to the endometrium.[12] Differences among reports may be due to the formulation studied in various countries and dosage, thus caution should be exerted when considering intrauterine enrofloxacin or, alternatively, enrofloxacin should be used systemically.

If we examine some of the more common antibiotics used in practice, one study found that 19% of beta-hemolytic Streptococcus isolates (includes *Streptococcus zooepidemicus*) were susceptible to gentamicin, whereas 96% of *Escherichia coli* isolates were susceptible.[10] In that study, 100% of the beta-hemolytic Streptococcal isolates were susceptible to ampicillin and penicillin G, whereas 86% of *Escherichia coli* isolates were susceptible to ampicillin. Another study evaluated intrauterine ceftiofur in mares and found that the drug had good antimicrobial activity and caused no increase in uterine inflammation when compared to controls.[21] Ticarcillin with clavulanic acid has been evaluated for intrauterine use and it was found that adequate intrauterine concentrations of the clavulanic acid portion are not maintained. Thus, this formulation may be questionable for intrauterine use.[22] These authors also reported that concentrations of ticarcillin declined rapidly after intrauterine administration, and multiple daily doses would be required.

Aminoglycosides have an acid pH that will irritate the endometrium.[3] It is suggested that aminoglycosides be buffered to a neutral pH with an equal volume of 7.5% sodium bicarbonate. Forty-three percent of practitioners added sodium bicarbonate, while 38% increased the volume of infusion as a means to moderate the acidic effects, and 10% did not add anything to the aminoglycoside. If saline is used to increase the volume of infusion to reduce the effect of low pH, it should be noted that saline has a pH of ≈5.5. A more suitable diluent may be lactated Ringer's solution which has a neutral pH.

Aminoglycosides should not be mixed with beta-lactam antibiotics. Precipitates may form when they are combined or, more importantly, aminoglycosides may cause a nucleophilic opening of the beta-lactam ring which then combines with an amino group from the aminoglycoside, resulting in a biologically inactive amide.[23,24] While the two drugs are synergistic in controlling Gram positive (beta-lactams) and Gram negative (aminoglycosides) infections when given systemically, it is not completely understood how effective they are when placed together into the uterine lumen. In addition, penicillin G (potassium or procaine) is inactivated by acids, so if penicillin and an aminoglycoside are used together in an unbuffered form, the penicillin may be less effective because of the low pH environment caused by the aminoglycoside. From survey results it appears that quite a few practitioners (34%) mix the two classes of drugs together either in the same syringe (20%) or the drugs are infused into the uterus at the same time (14%). For maximum effectiveness, mixing these drugs within the uterus should be discontinued and the drugs should be given either systemically or their administration separated in time by an unknown number of hours if given by intrauterine infusion. It is also not recommended to mix the two classes of drugs in lavage solutions. Interestingly, there are many semen extenders that combine potassium penicillin and amikacin. This practice may diminish the effectiveness of the antibiotics. Conversely, gentamicin has a high rate of inactivation when mixed with certain beta-lactams, while amikacin is only slightly inactivated.[25]

When asked about which antifungal intrauterine drug they used prior to receiving culture results, the majority of practitioners (32%) would not use an antifungal drug, but instead opted for either a povidone-iodine solution lavage, lufenuron or, less commonly, a dilute vinegar lavage. If an antifungal drug were used, then the most common responses included clotrimazole (17%), nystatin (11%), miconazole (10%), fluconazole (8%) and amphotericin B (3%). No answer was provided by 19% of the respondents. Only 53% of practitioners submitted fungal cultures for sensitivity assay. Lack of antibiotic sensitivity patterns to determine the most appropriate therapy may explain, in part, why fungal uterine infections are difficult to treat. The reasons stated for not submitting fungal cultures for a sensitivity are: length of time to receive results; had success with povidone-iodine lavage; all seem sensitive to amphotericin B; inability to obtain fungal sensitivities from the laboratory; the relative infrequency with which fungal infections were encountered precluded sensitivity testing; just treated *Candida* infection with nystatin; or treating seems to work just fine. There are a number of laboratories that offer fungal sensitivity patterns including the laboratory at Cornell University. There was also concern from practitioners that in vitro sensitivity patterns may not correlate with in vivo effectiveness. It would appear that within the group of polyene antifungal antibiotics, amphotericin B (96% susceptibility of all fungal organisms) and nystatin (100% susceptibility) are good choices, where as clotrimazole (80% susceptibility) or ketoconazole (81% susceptibility) are good choices when using azole antifungal antibiotics. (personal communication, Marco Coutinho da Silva, Cornell University) Polyene antibiotics are generally considered fungicidal, whereas azole antibiotics are fungistatic, except at higher doses. Some practitioners try to avoid intrauterine antifungal treatments with the concern that repeated intrauterine

treatment may make the mare more susceptible to re-infection or prolonged inflammation. An alternative would be oral antifungal drugs which may be expensive.

Lufenuron is a chitin inhibitor which has been used in an extra-label manner for treatment of fungal uterine infections.[26] It should be noted that lufenuron affects the wall of growing fungi and may not be appropriate for treatment of mature infections. A better approach may be to treat with an antifungal antibiotic and then at the end of treatment, place lufenuron in the uterus to prevent new growth. The effectiveness of lufenuron still remains in question.[27,28]

Uterine lavage with either iodine or vinegar is a component of therapy for many veterinarians when treating fungal infections. Forty percent of veterinarians used a dilute iodine solution for lavage (24% added iodine to saline, 16% added iodine to lactated Ringer's solution). The percent iodine in lavage solutions in the survey range from 0.02% (2 ml of 10% iodine per liter) to 0.5% (50 ml of 10% iodine per liter). A 0.2% solution of iodine infused into the uterus has been associated with endometrial inflammation and fibrosis.[29] A 0.01% to 0.05% solution of iodine maintains antimicrobial activity[30] without causing inflammation and fibrosis.[31] Practitioners should be cautious of the higher concentrations of iodine in intrauterine infusions. Twenty-two percent of veterinarians used a dilute vinegar solution (15% of practitioners added vinegar to saline, 7% added it to lactated Ringer's solution). When using vinegar, saline would be a more appropriate lavage solution, if the desire is to lavage with a lower pH solution. Addition of 20 ml of white vinegar to 1000 ml of saline (2% v:v solution) will reduce the pH from ≈5.5 to ≈3, whereas it has little effect on the pH of lactated Ringer's solution.

Intrauterine antibiotics and lavage should be avoided within 4 hours of breeding[12] so that spermatozoa are not negatively affected by the drugs or the vehicles in which they are delivered. After 4 hours post-insemination, spermatozoa are located in the oviduct and intrauterine treatment at this time does not have a negative effect on fertility.[32] Most practitioners who participated in the survey appeared to be aware of this, with only 16% of them infusing antibiotics within 4 hours post-insemination. Most practitioners (37%) withheld treatment for more 4 hours post-breeding, because the next examination, and thus treatment of the mare, did not occur until the day following insemination.

Systemic antibiotics

The decision to use systemic antibiotics either in combination with intrauterine infusion, after intrauterine infusion or instead of intrauterine infusion of antibiotics may be due to personal preference, a desire to prolong the treatment period, because the organism is not susceptible to non-irritating drugs, or to avoid manual manipulation of the reproductive tract. Results of the on-line survey indicated that systemic antibiotics are chosen when intrauterine treatments extend beyond 3 to 5 days, when treating mares with metritis, when treating mares with contaminated caudal reproductive tracts, when treating mares with anatomical defects of the caudal reproductive tract or occasionally when treating mares with fungal infections. Respondents felt that systemic antibiotics negate the need to invade the uterus, possibly avoiding the chances of iatrogenically placed bacteria or fungi. The disadvantages of using systemic antibiotics are increased costs and inconvenience from having to dose at the animal's full body weight and possibly the need to treat multiple times per day. A very small number of practitioners felt that it was not good veterinary practice to place antibiotics directly into the uterus, since systemic antibiotics work well, do not cause endometrial irritation, and do not lead to further contamination.

Dosages for antibiotics commonly used systemically are presented in Table 2. Trimethoprim sulfadiazine, ceftiofur, and a combination of penicillin and gentamicin were the most common antibiotics administered by practitioners who participate in the survey. Trimethoprim sulfamethoxazole (30 mg/kg, per os, q12h) was found to provide adequate antibiotic concentrations in fetal tissues in mares with placentitis.[33] In a separate study, ceftiofur dosed at 2 mg/kg q12h intramuscularly, did not result in adequate endometrial tissue levels;[34] however, it has been suggested as a potential treatment for mares with placentitis.[35] Higher dosage concentrations (recommended up to 4.4 mg/kg) and/or intravenous treatment could perhaps result in adequate endometrial levels. In cattle, minimal inhibitory concentrations of ceftiofur are achieved in endometrial tissue after subcutaneous administration.[36] A study by Murchie, et al., found that intravenous administration of penicillin G potassium and gentamicin sulfate resulted in adequate allantoic fluid concentrations in pregnant pony mares.[37] Enrofloxacin has also been used in mares with more resistant bacteria.[20,38,39] Enrofloxacin should not be used in pregnant mares due to its effects on developing cartilage.[40] Doxycycline has also been demonstrated to reach endometrial concentrations above the minimum inhibitory concentration for *Streptococcus equi* subspecies *zooepidemicus*.[41]

Antifungal antibiotics may be administered systemically. Amphotericin B is fairly caustic due to a low pH and needs to be given via nasogastric intubation or diluted and given slowly intravenously. Oral fluconazole has been recommended for treatment of *Candida* spp. while oral itraconazole has been suggested for treatment of *Aspergillus* spp. (personal communication, Marco Coutinho da Silva, Cornell University).

Uterine Cytology

Uterine cytology was performed in conjunction with 67% of uterine cultures. This is a relatively easy procedure to perform and interpret.[42] Sixty-four percent of practitioners either read their own (56%) or had someone in their practice (8%) read cytologies. By performing this examination "in-house" results may be interpreted and therapy instituted without the delay of sending the slides to an outside laboratory. Only 9% of practitioners, however, used Gram stain to distinguish Gram negative from Gram positive bacteria in order to institute appropriate antimicrobial therapy.

Treatments to augment antimicrobial therapy

Uterine lavage is recommended to remove uterine debris, bacteria and fungi and to enhance uterine contractility. DMSO lavages may be useful to augment tissue penetration and to disrupt microbial biofilms.[43] Acetylcysteine and kerosene have also been suggested as possible mucolytic agents. Biofilms are aggregates of bacteria and/or fungi encased in an adherent polymeric matrix which may inhibit antibiotic penetration.[44-46] Biofilms have been known to form with *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia*, and *Candida* spp.[47]

Tris-EDTA has been demonstrated to act synergistically with antimicrobials by increasing the membrane permeability of bacteria to these drugs.[48] Uterine lavage with tris-EDTA, either alone or in combination with antibiotics, should be considered with resilient infections or in cases with antibiotic-resistant organisms.

New intrauterine therapies

Intrauterine foam (Fatroximin®; Fatro, Bologna, Italy) containing the antibiotic rifaximin, a synthetic derivative of rifamycin, has been developed for use in cattle and horses.[49] It has a spectrum of activity that includes Gram negative, Gram positive and anaerobic bacteria. The drug, in the foam vehicle, has a 72+ hour residual effect and expands to cover the entire uterine lumen. A single treatment is recommended for treatment of endometritis or the product may be administered for two consecutive days for treatment of vulvovaginitis. This drug is currently available in Europe.

Conclusions.

Veterinarians should base antibiotic therapy on sensitivity tests. Consideration should be given to antibiotic therapy alternatives such as proper breeding management, use of uterine lavage and oxytocin or prostaglandin treatment. Biofilm formation should be appropriately treated to enable antibiotics to access bacteria and fungi. With a plan, antibiotic usage can be minimized and treatment success optimized. The main issues of concern identified from the on-line survey of veterinarians are: mixing beta-lactam and aminoglycoside antibiotics for intrauterine infusion, intrauterine infusion of high concentrations of iodine solutions, use of lactated Ringer's solution with vinegar for uterine lavage, and treatment of mares less than 4 hours post-breeding with intrauterine antibiotics.

Table 1. Intrauterine antibiotic dosages.

Intrauterine antibiotics			
Antibacterial antibiotics			
Antibiotic	**Dosage**	**Comments**	**Major bacterial susceptibility**
Amikacin	1 to 2 grams	Buffer with sodium bicarbonate or 150 to 200 ml solution	Gram negative
Ampicillin	1 to 3 grams	Use soluble product, may be irritating when concentrated	Gram positive and *E.coli*
Ceftiofur sodium	1 gram		Gram positive and Gram negative
Chloramphenicol	2 to 3 grams	Can be irritating	Gram positive and Gram negative
Gentamicin	1 to 3 grams	Acidic – need to dilute and/or buffer	Gram negative
Neomycin	2 to 4 grams		Gram negative
Potassium penicillin	5 million international units		Gram positive

Procaine penicillin	4.5 to 6 million international units	Concern about residue left in uterus	Gram positive
Ticarcillin	3 to 6 grams	Infuse with 150-200 ml solution	Gram positive, *Pseudomonas*
Ticarcillin with clavulanic acid	3 to 6 grams	Beta-lactamase inhibitor, infuse with 150-200 ml solution	Same as ticarcillin plus more Gram positive (*Staph, Bacillus, Enterobacter*)
Antifungal antibiotics q24h for 7 days			
Drug	**Dosage**	**Comment**	
Amphotericin B	100-200 mg	Polyene, dilute in >100 ml solution, mix well	
Clotrimazole	400 to 700 mg	Azole, tablets usually crushed and mixed with solution	
Fluconazole	100 mg	Azole, may need to adjust pH to avoid acidic nature	
Miconazole	500-700 mg	Azole	
Nystatin	0.5 to 2.5 million international units	Polyene, Dilute in sterile water, not saline to avoid precipitates, mix well	

Table 2. Systemic antibiotic dosages. (IV-intravenous, IM-intramuscular, PO-per os, q-every, h-hour, IU-international units, kg-kilogram)

Systemic Antibiotics			
Antibacterial antibiotics			
Drug	**Dosage**	**Route, Comment**	
Amikacin	10 mg/kg q24h	IV or IM	
Ampicillin	29 mg/kg q12-24h	IV or IM	
Ceftiofur	2 to 4 mg/kg q12h	IV or IM	
Doxycyline	10 mg/kg q12h	PO	
Enrofloxacin	5.5 mg/kg q24h	IV	
	7.5 mg/kg q24h	Per os	
	4.0 mg/kg q12h	Per os	
Gentamicin	6.6 mg/kg q24h	IV or IM	
Metronidazole	15 to 25 mg/kg	PO	
Oxytetracycline	6.6 mg/kg q12h	IV, dilute and give slowly	
Potassium Penicillin	22,000 IU/kg q6h	IV	
Procaine Penicillin	22,000 IU/kg q12h	IM, only 10 ml per injection site	
Trimethoprim Sulfa	30 mg/kg q12h	PO	
Antifungal antibiotics			
Drug	**Dosage**	**Route**	**Comments**
Amphotericin B	0.3 to 0.9 mg/kg q24-48h	IV	Polyene, dilute and give slowly
Fluconazole	14 mg/kg loading, then 5mg/kg q24h; alternatively, 2 grams q24h	IV or per os	Azole
Itraconazole	5 mg/kg q12-24h	IV or per os	Azole, oral suspension more bioavailable than capsules
Ketoconazole	20 mg/kg q12h in 0.2 N HCl	Per nasogastric intubation	Azole, irritant if given per os due to low pH – need to place into stomach

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New treatment strategies for chronic endometritis and post mating endometritis

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Abstract

Traditional treatments for chronic endometritis and post-mating induced endometritis including intra-uterine antibiotics, uterine lavage and ecbolics do not always resolve an infection or clear uterine fluid. Treatment failure may be due to continual contamination of the uterus because of anatomical abnormalities in the caudal tract, degradation of antibiotics in uterine exudate, biofilm production by micro-organisms or prolonged uterine inflammation. Older, pluriparous mares are most commonly affected as they are unable to physically clear uterine contamination or inflammation after breeding. Nulliparous mares may also develop persistent mating induced endometritis or chronic endometritis if they have an incompetent cervix as it will prevent rapid drainage. Repeatedly treating chronically infected mares with intra-uterine antibiotics can lead to multi-drug resistant infections while prolonged inflammation in mares with post mating induced endometritis can eventually result in bacterial or yeast endometritis. Because traditional treatments are not always successful, a number of agents and treatment strategies have been investigated. These include buffered chelators that potentiate antibiotics (tris-EDTA), mucolytics (DMSO, kerosene, n-acetylcysteine), corticosteroids (prednisolone, dexamethasone) and immunomodulators (cell wall extracts of *Mycobacterium phlei* and *Propionibacterium acnes*). All have shown some degree of success when cases are selected carefully and protocols are followed.

Keywords: Mare, endometritis, chelating agents, mucolytics, immunomodulation

Introduction

Traditional therapy for chronic endometritis includes removal of the offending organism through uterine lavage, judicious use of ecbolics and antimicrobial therapy for three to five days during estrus in addition to repair of anatomical defects.[1,2] Uterine irrigation and administration of oxytocin or cloprostenol within eight hours of mating followed by a second treatment at 24 hours that may or may not include intra-uterine antibiotics, is a recommended protocol for post mating induced endometritis.[3,4] However, these protocols are not always successful in clearing uterine fluid or infection. Treatment failure may be due to an inability to physically clear uterine fluid quickly after mating, continual production of uterine fluid secondary to inflammation, or an inability of antibiotics to penetrate exudate or biofilm produced by microorganisms. Because treatments have failed, intrauterine buffered chelators (tris-EDTA; ethylene-diamine tetra-acetic acid (3.5 M)-tromethamine 50mM; Rood and Riddle Veterinary Pharmacy, Lexingon, KY, USA and Tricide®; 8mM disodium EDTA dehydrate and 20 mM 2-amino-2-hydroxymethyl-1,2-propanediol; Medical Molecular Therapeutics, LLC, Athens, GA, USA), mucolytics (DMSO, kerosene, n-acetylcysteine), corticosteroids (prednisolone, dexamethasone) and immunomodulators (cell wall extracts of *Mycobacterium phlei* and *Propionibacterium acnes*) have been investigated and have shown potential as effective therapies for endometritis if used appropriately. Some of these agents offer alternatives to repeated use of anti-microbial agents, which is often the major instigating factor for antibiotic resistance. Clinical studies on large groups of barren mares are lacking though and need to be performed before true efficacy can be determined.

New treatment strategies for chronic endometritis

The most critical factor in uterine defense against infection is rapid, physical clearance of inflammatory debris from the uterus after mating or post foaling. Some mares have difficulty clearing this debris because they have developed anatomical and/or degenerative defects that interfere with uterine drainage. Repeated foaling and breeding can cause anatomical defects such as poor perineal conformation, incompetent vagino-vestibular sphincter, vaginal stretching, incompetent cervix, a pendulous uterus or degenerative changes such as an abnormal myometrium, periglandular fibrosis, vascular elastosis, lymphangectasia, scarring and atrophy of endometrial folds or damage to the mucociliary apparatus. Older nulliparous mares that are not mated until 10 or more years of age and those that have repeated embryo recovery attempts also experience delayed uterine clearance, often because of cervical malfunction.[4-8] The uterus responds to prolonged retention of inflammatory debris by increased mucus production by epithelium, transudation of serum proteins, and an influx of neutrophils and immunoglobulins into the uterine lumen. If these substances remain in the uterine lumen for more than 24 to 48 hours, endometrial ulceration and secondary bacterial infections may result.[1,9-11] Bacterial endometritis is most commonly treated with intra-uterine therapies (i.e. uterine lavage, ecbolics and intra-uterine antibiotics). Most uterine infections resolve after a three to five day course of antibiotics as long as inflammation is not severe, antibiotics are not rendered ineffective and anatomical defects do not compromise the mare's ability to physically clear the uterus of bacteria, inflammatory debris and contaminants. However, if uterine degeneration is severe, the cervix is fibrotic, or the offending

organism produces a biofilm, treatment with intra-uterine antibiotics can lead to secondary fungal endometritis or infection with multi-drug resistant bacteria.

Multi-drug resistant bacteria have been isolated from the uterus of mares after repeated intra-uterine antibiotic treatment including methicillin resistant *Staphylococcus aureus* and multi-drug resistant *Pseudomonas aeruginosa, Staphylococcus epidermis, E. coli,* and *Enterobacter cloacae* (personal communication, Marianne Swintosky, 2008). These findings and the wider implications of antibiotic resistance in humans support development and use of novel strategies to combat equine uterine infections.

Mucolytics

Mucus plays an important role in protecting and cleansing of mucosal surfaces such as the respiratory and gastrointestinal tract.[12] It may have a similar role in the reproductive tract as the equine endometrium contains cilia and is covered by a mucus blanket.[13,14] Mucus production at the equine endometrial surface has been demonstrated using alcian blue,[14,15] mucicarmine[16] and periodic acid Schiff stains.[14,17] Excessive mucus production by the equine endometrium, detectable in uterine lavage fluid[18] or uterine biopsy specimens,[14,17] is now linked to failure to become pregnant. During acute and subacute uterine inflammation there is an increase in mucus production and in the height of epithelial cells.[17]

Solvents and mucolytic agents have been added to uterine irrigation fluids in an attempt to clear exudate, mucus or biofilm. Agents used include DMSO, kerosene and N-acetylcysteine (20% solution; Butler Corp, Columbus, OH, USA). Each compound appears to have some beneficial effects. Barren mares (n = 16) infused with a 30% solution of DMSO after breeding tended to have higher pregnancy rates than mares infused with saline.[19] Intrauterine DMSO therapy also resulted in a significant improvement in endometrial biopsy classification in 18 of 27 mares; whereas only 2 of 18 barren mares improved following intrauterine saline treatment. In contrast, intrauterine infusion of 50 ml of commercially available kerosene in 26 mares with varying degrees of endometrial pathology induced diffuse moderate to severe endometritis, severe diffuse edema and production of a serum-like exudates.[20] Half of the mares exhibited mild to severe necrosis of luminal epithelium. Mares were subsequently bred on the next cycle and surprisingly, 50% of the mares with Category II or III biopsy scores carried foals until term. Although kerosene was associated with significant inflammatory changes, pregnancy may have been established because mucus and exudate were removed via destruction and necrosis of uterine epithelium.

N-acetylcysteine (NAC) is a mucolytic agent that disrupts disulphide bonds between mucin polymers, thereby reducing the viscosity of mucus. In addition, NAC possesses antioxidant and possibly some antimicrobial properties.[21-23] NAC has been used to treat respiratory diseases such as pneumonia, the pulmonary component of cystic fibrosis in humans, meconium impactions in both humans[24,25] and equine neonates (Morresey PR, personal communication, 2008), and meconium aspiration pneumonia in equine neonates.[26] Multiple studies support its beneficial anti-oxidative properties especially in chronic inflammatory diseases.[21-23,27] We have recently evaluated its effect on the endometrium and epithelium.[28] Endometrial biopsies were obtained from fertile and barren mares before and after infusion of a 3.3% solution of N-acetylcysteine (day 1) and compared to biopsies obtained from mares infused with saline. The uterus of all mares was irrigated with 2 L of lactated Ringer's solution on days 2 and 3 and a second biopsy obtained. Endometrial biopsies were given a Kenney grade by a board certified veterinary pathologist and changes in epithelial architecture and mucus blanket were measured by image analysis. Data indicated that NAC was not harmful to the endometrium and that it may counteract the irritating effect of saline, as reflected through increased cell height in control mares. As further evidence that NAC does no harm and may be beneficial, 20 barren Thoroughbred mares bred 2 to 5 times in 2007 or 2008 and with a history of endometritis were mated naturally to commercial stallions in Central Kentucky in late May and June 2008. Mares received a 0.6% solution of NAC either the treatment cycle before (n = 10) or in the 48 h before breeding (n = 10) in addition to conventional treatments. Infusion before breeding was associated with higher than expected pregnancy rates as 17 of 20 mares (85%) were pregnant as of February, 2009. Prior to this study, the rationale for using NAC as a uterine infusion had been the removal of inspissated secretions, exudate and biofilm, (i.e. as a mucolytic). However, since increased vaginal mucus viscosity is documented to inhibit sperm forward progression in cows,[29] it is also speculated that NAC may improve sperm-transport in mares with excessively viscous mucous secretions by breaking the cross-linking disulfide bridges between mucin polymers.

Bacterial and yeast biofilms

Antibiotic failure in chronic endometritis may be due to biofilm produced by some gram negative bacteria, yeast and fungi. Bacterial biofilms consist of a heterogeneous community of different bacterial species, surrounded by an extracellular matrix, that co-exist in a symbiotic relationship.[30] Such biofilms are found throughout the human body, e.g. the oral cavity, the skin, the intestines and the vagina. In most cases, the inhabitants of this community are considered as normal flora and serve as a protective mechanism to prevent the colonization of frank and opportunistic pathogens. If the balance of this biofilm community is upset or disrupted, pathogens may colonize,

proliferate, and cause disease.[30] Biofilms confer antibiotic resistance and therefore contribute to treatment failure. A number of theories have been advanced to account for this increased resistance.[31-34] One is simply that the antibiotic is unable to penetrate the extracellular matrix of the biofilm. Another is that antibiotics are less active on biofilms due to the lower rate of metabolism and growth. A currently popular theory is that there are "persister cells" within the biofilm community. Persister cells are defined as a small subpopulation of essentially invulnerable cells that neither grow or die in the presence of bactericidal agents and exhibit multi-drug tolerance or resistance to antibiotics.[30]

Pseudomonas aeruginosa is a potent biofilm producer and is often cultured from the uterus of mares with chronic endometritis. Other equine pathogens that produce biofilm and can be isolated from the uterus include *Staphylococcus epidermis, E. coli, Enterobacter cloacae* and a number of yeast and fungi. These organisms more commonly cause endometritis in older, pluriparous barren mares that have anatomical defects than young, fertile mares, although uterine defenses can be broached in the latter resulting in chronic infection. Infections by these organisms can be difficult to treat, are often refractory to a 3 to 5 day course of antibiotics, and may result in a population of bacteria colonizing the uterus that is highly resistant to the drug initially used for treatment. Work in other species and in the mare have been shown that buffered chelating agents (tris-EDTA) may potentiate the actions of antimicrobials, dissolve exudate, and break up biofilm.

Buffered chelators such as first generation tris-EDTA[35-41] and third generation Tricide® potentiate the actions of antimicrobials.[42] They have been shown to enhance the bactericidal effects of antimicrobials in dogs with refractory otitis,[35,37,39] pyoderma,[39] osteomyelitis,[36] multiple fistulas,[36,43] rhinitis,[44] and cystitis.[39,45] Uterine isolates of *Pseudomonas* collected from mares exposed to tris-EDTA solution exhibited decreased viability.[46] Others have shown that addition of tris-EDTA to gentamicin *in vitro* improved killing of *Pseudomonas aeruginosa* by 1000 fold more than treatment with only gentamicin.[47] Addition of tris-EDTA to penicillin, ampicillin, oxytetracycline, neomycin, and amikacin has also been shown to be synergistic.[42] A recent study showed that Tricide® increased *in vitro* activity of antifungal drugs against common fungal pathogens isolated from eyes of horses with mycotic keratitis.[42] The mechanism of action of buffered chelating agents is not completely understood but it is speculated that the chelating agent (EDTA) chelates calcium and/or magnesium from the outer membrane of bacteria, thereby altering the integrity and permeability of the cell wall. Damage to the cell wall interferes with the effectiveness of the bacterial efflux pump and facilitates osmotic collapse. Unlike bacteria, fungal cell walls are composed mainly of polysaccharides (beta-glucans and chitin) and protein. It is hypothesized that removal of divalent cations in the cell wall by third generation chelating agents may alter membrane proteins that are important in maintaining the construction and maintenance of the polysaccharides in the wall.[42]

Buffered chelators reportedly have minimum adverse effects when used in joints,[36] bones,[36] the uterus,[48] ears,[35,37,41] the bladder,[39,45] and mammary glands.[36] Treatment with tris-EDTA, a first generation chelating agent, appears not to be harmful as infusion of 250 ml of 3.5 M EDTA, 0.05 M tris, pH 8, into the uterus induced an inflammatory response that was no greater than saline.[48]. The benefit of third generation chelating agents such as Tricide® over first generation chelating agents is greater antibiotic stability in third generation chelating solutions (B.W. Ritchie, personal communication, 2009). There are no clinical studies on the use of third generation chelating agents in the treatment of bacterial or yeast endometritis.

Buffered chelating agents must come in direct contact with the bacterial cell wall in order to kill the organism so the volume of solution needed for infusion will vary with the size of the uterus. Doses ranging from 200 to 500 ml are recommended. The chelating agent binds to the bacteria within minutes resulting in cell death and accumulation of debris so the uterus should be lavaged within 12 hours to remove these by-products (B.W. Ritchie, personal communication, 2009).

New treatment strategies for post mating induced endometritis

Fluid may accumulate within the uterine lumen during estrus because it is not physically drained through the cervix, or production is increased secondary to chronic inflammation, bacterial infection or vestibule-vaginal reflux. Degenerative uterine changes such as vascular elastosis may also contribute to fluid accumulation. Vascular elastosis appears to indirectly reduce fertility through a reduction in endometrial perfusion, and through disturbances in uterine drainage caused by reduced venous return in capillary beds.[49-51] For the past 20 years, treatment of post mating induced endometritis has emphasized methods for improving physical drainage. The currently recommended therapy for improving physical clearance of uterine fluid is uterine irrigation followed immediately by administration of either oxytocin (10 to 25 IU i.v. or i.m.) or cloprostenol (250 μg i.m.) at 4 to 8 hrs after breeding.[4,52-58] This treatment has increased pregnancy rates in highly susceptible barren mares.[59] A long-acting synthetic oxytocin analog, carbetocin, has recently become available in Europe, Canada and Mexico. It was well-tolerated in a group of horses following intravenous administration of 175 μg. The half-life of carbetocin is about

17 minutes, or 2.5 times that of oxytocin.[60] The drug may be of benefit in mares where more prolonged uterine contractions are needed. No clinical studies comparing its efficacy with oxytocin have been reported.

In a mare with a cervix which fails to dilate, such as an aged maiden mare, oxytocin may be ineffective in expulsion of uterine fluid. However, similar to its role in promoting lymphatic drainage, cloprostenol may help to expel uterine fluid through a narrow cervix through sustained uterine contractions. In addition, the cervix may be manually dilated to assist fluid drainage. We have used a compounded misoprostol product (2000 µg/3 ml; Rood and Riddle Veterinary Pharmacy, Lexington, KY, USA), a synthetic prostaglandin E1 analog, that clinically appears to have resulted in cervical relaxation when applied topically to the cervical epithelium 2 to 4 h before breeding.

There is a clinical impression that oxytocin does not always effectively clear uterine fluid in old, pluriparous mares so cloprostenol is frequently given in place of oxytocin. However, cloprostenol has been shown to be associated with a decrease in serum progesterone concentrations if given after ovulation.[61,62] Because of perceived treatment failures, complications with administration of cloprostenol and the fact that retained uterine fluids contain inflammatory by-products that adversely affect embryo viability; modulation of the immune system has been investigated.

Recent work has shown that steroids or immunomodulators administered judiciously around the time of mating may increase pregnancy rates in mares with fluid accumulation or uterine inflammation.[63-69] Immunomodulation by either administration of steroids or immunomodulators may help restore homeostatic local inflammatory mechanisms through reducing pro-inflammatory cytokines. This may be especially helpful in older mares that may be suffering from inflamm-aging. Inflamm-aging is a low-grade, systemic inflammatory response associated with advanced age in humans and horses that is characterized by increased inflammatory cytokine production.[70,71] Peripheral blood mononuclear cells collected from old horses have been shown to produce more inflammatory cytokines than mononuclear cells from young horses; moreover, fat old horses have even greater frequencies of lymphocytes and monocytes producing inflammatory cytokines than thin old horses. Weight loss in old fat mares reduced the percent of IFNγ and TNFα positive lymphocytes and monocytes and serum levels of TNF α protein. When weight and fat increased in these old horses, there was a significant increase in inflammatory cytokine production.[70,71]

A single dose dexamethasone administered within one hour of mating and daily prednisolone administration given before and after mating have improved pregnancy rates in mares with uterine fluid. Bucca, et al.[67] reported that a single injection of dexamethasone administered within one hour of mating (50 mg, IV; approximately 0.1 mg/kg) combined with routine post breeding therapies (uterine irrigation, ecbolic drugs and in some cases intra-uterine antibiotics) resulted in increased pregnancy rates in mares with a history of fluid accumulation after ovulation and in mares with cervical incompetence. Treated mares exhibited decreased uterine edema, decreased intrauterine fluid and an increase in uterine fluid clarity. Although dexamethasone did not increase pregnancy rates in the general population, pregnancy rates were increased in mares that had 3 or more risk factors for susceptibility to endometritis. Risk factors included abnormal reproductive history, abnormal perineal conformation, vulvoplasty not repaired after foaling, an incompetent cervix, positive endometrial culture, $\geq$ 2 cm of endometrial fluid before breeding, endometrial fluid post mating between 1.5 and 2.0 cm, or a fluid volume $\geq$ 2cm, and endometrial fluid persisting more than 36 hours after mating. Increased pregnancy rates were also observed in mares with a history of intra-uterine fluid accumulation following oral administration of acetate 9-alpha-predinisolone (0.1 mg/kg) given at 12 h intervals for 4 days beginning 48 hrs before breeding.[66] In contrast, administration of dexamethasone (10 or 20 mg, IM) 6 to 12 h after insemination did not improve pregnancy rates of warmblood mares with a history of intra-uterine fluid retention (n=783 cycles).[69] A plausible cause for the different results is that steroids block both the cyclooxygenase and 5-lipoxygenase pathways of inflammation. The 5-lipoxygenase pathway includes leukotriene B, a potent neutrophil chemotactic factor found in uterine fluids of susceptible mares after mating.[72,73] Reducing neutrophil chemotaxis and the number of neutrophils recruited into the uterus post mating may diminish the severity and length of the inflammatory response. Candidates for steroid use should be chosen carefully as misuse in mares with bacterial endometritis may exacerbate the infection.

Immunomodulators may also improve pregnancy rates, although the mechanism of action remains speculative. Immunomodulators induce a nonspecific cell-mediated response predominantly by activation of macrophages and release of cytokines that elicit a general increase in immune system activity.[74] Two immunomodulators are currently labeled and marketed for use in horses. One is a cell-wall extract of *Mycobacterium phlei* (MCWE; Settle®, Bioniche Animal Health, Bogard, GA, USA) that has been approved as an adjunctive treatment in mares with uterine infection caused by *Streptococcus equi* subspecies *zooepidemicus*. Studies have shown that it modulates the immune response of susceptible mares[63,64] and that mares with experimentally induced bacterial endometritis cleared inflammation more rapidly after treatment with MCWE compared to untreated mares.[65] The second immunomodulator is *Propionibacterium acnes* (EqStim®, Neogen

Corp, Lexington, KY, USA). It is used as an adjunct treatment for horses with equine respiratory disease complex. Pregnancy and live foal rates were higher in barren mares with a cytologic diagnosis of persistent endometritis treated with both *P. acnes* and conventional treatments than in mares treated only with conventional treatments. [68]

Conclusion

Novel treatment strategies for chronic endometritis or persistent mating induced endometritis have been recently evaluated in mares. Treatments for chronic endometritis include adding chelating solutions to potentiate antibiotics, irrigating with mucolytics to dissolve excessive mucus or biofilm and adding oxygen radical scavengers to irrigation solutions to reduce inflammation. Although improving physical uterine clearance after mating will remain the primary treatment for mares with persistent post mating induced endometritis, administration of immunomodulators around the time of mating has been shown to improve pregnancy rates.

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Treatment strategies in the perinatal mare and foal

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Abstract

A large number of conditions are recognized in the peripartum period that have the potential to impact the health and future performance of both the mare and the foal. Peripartum hemorrhage can be insidious in onset and result in profound abdominal pain, hypovolemia and in severe cases death of the mare. Those mares that survive the initial hemorrhage may succumb some time after the apparent stabilization of the hematoma. Treatment is aimed at promoting hemostasis and improving cardiovascular performance while not promoting further hemorrhage. Many drugs in common use have been chosen due to anecdotal reports of success or extrapolation from studies on horses or other species. Large, multi-center controlled trials of therapeutic agents are lacking, this being expected given the critical nature of the condition. The compromised neonatal foal is a challenge to the attending clinician, who must balance the immediate medical needs of the foal with the financial requirements of the client. Following the initial assessment, resuscitation of the neonate must be performed in a timely and efficacious fashion. The attending clinician should establish a coherent and consistent approach to cardiovascular resuscitation of the foal. Following this event, stabilization of the neonate and preparation for transport to a referral center if required can occur. A thorough knowledge of the expected neonatal development of the foal will aid in selection of foals requiring specialist intervention.

Keywords: Mare, peripartum, hemorrhage, foal, neonatal, resuscitation

Introduction

The peripartum period for both the mare and the foal can be a period of great risk to life and future potential. A myriad of problems exist, and it is beyond the scope of this presentation to cover them all. Therefore a selected few will be covered in detail: peripartum hemorrhage in the mare, neonatal foal resuscitation, stabilization and preparation for referral.

Mare

Considerations in the periparturient mare

The periparturient mare does not differ substantially from the non-pregnant mare with respect to general husbandry. Routine exercise, a balanced diet and regular preventative health care (teeth, deworming and vaccination) should all continue as before.

Differences become important when they involve the overall health of the mare and the effects on placental function that systemic illness can have. The goals of therapy should address resolution of the precipitating problem, systemic support for the mare (and therefore fetoplacental unit) and the avoidance of fetal hypoxia.

The overall health of the mare will be affected by any disease process resulting in inappetance, fever or proinflammatory mediator production with systemic release. Nutritional insult resulting in weight loss during mid-gestation has detrimental effects on placental development which results in reduced fetal growth.[1] Endocrine function of the foal is also affected.[2]

Colic is also of greater concern in the periparturient mare, as this condition presents a serious diagnostic challenge to the practitioner through limitations imposed upon examination by the presence of the gravid uterus. Accurate differentiation between gastrointestinal conditions and gestational accidents is essential but difficult in many cases. Early gastrointestinal problems can appear similar to initial stages of labor. Peripartum gastrointestinal conditions include large colon volvulus or displacement, cecal rupture, small intestinal volvulus, enterocolitis and direct trauma to the intestine.[3]

Trauma to the gut may result in ischemic necrosis of the affected areas.[4-6]. Compromise to the gastrointestinal tract results in the onset of endotoxemia with profound circulatory dysfunction and proinflammatory stimuli occurring. Serious metabolic insults can affect both the mare and concurrently the fetus. Treatment of the gastrointestinal disease may involve general anesthesia with abdominal exploration further insulting the fetoplacental unit.

Hypoglycemia of the mare resulting from inappetance or fasting while colic treatment continues decreases glucose delivery to the fetus. Administration of glucose to late-term pregnant mares is recommended to avoid this, as is supplementation of feed material by nasogastric intubation where practical. Oxygen therapy is also recommended where placental function is suspected to be compromised.[7]

Reproductive problems in the peripartum period with apparent colic as a presenting clinical sign include uterine torsion, uterine rupture, uterine laceration, uterine bruising and hemorrhage of the blood vessels supplying the reproductive tract.[3]

Peripartum hemorrhage

Rupture of and subsequent hemorrhage from the uterine artery is the most common cause of death in mares *post partum*.[3] The external iliac artery, utero-ovarian artery, and uterine artery have also been implicated.[8] In a review of central Kentucky mares, reproductive complications accounted for the majority (57 of 98 cases, 58%) of deaths in peripartum mares.[9] Of those that died of reproductive complications, rupture of a uterine artery was determined to be the cause of death in 40 cases (70%). The incidence of peripartum hemorrhage in the mare has not been determined by retrospective studies of large numbers of mares, instead reports of clinical cases are found in the literature. Hemoperitoneum itself is a significant cause of abdominal discomfort in the horse, with approximately 13% of all cases due to rupture of uterine vessels.[10]

Although usually considered a problem of the post partum period, a number of reports exist of cases in the prepartum period.[8,11] Peripartum hemorrhage has been reported to occur at any age, however older mares are considered to be at greater risk.[12] Age-related degeneration of arterial vessels associated with the reproductive tract is suspected to be the reason for the increased incidence in older mares, coupled with the increased mechanical stresses imposed by the gravid uterus. Uterine contractions and obstetrical manipulations further increase stress on the vessel wall.[13] Copper deficiency was identified as a contributing factor in mares experiencing fatal hemorrhage, whereas non-fatal hemorrhage mares had comparable copper levels to their non-affected cohorts.[14]

Peripartum hemorrhage can occur in any of the following forms: hemorrhage into the peritoneal cavity, hemorrhage retained either within the broad ligament of the uterus or within the uterine wall (mural hemorrhage) or hemorrhage into the uterine lumen. Combinations of these may occur, necessitating thorough evaluation of mares affected by seemingly less serious forms of hemorrhage so as to avoid non detection of life-threatening episodes.

Hemorrhage into the peritoneal cavity can lead to profound hypovolemia, pain and result in peracute death. If confined to the broad ligament or uterine wall, pain can still be significant but prognosis for life is better. These hematomas may be incidental findings during routine reproductive examinations, or may become acutely apparent some time after foaling following the onset of abdominal hemorrhage. Hemorrhage within the uterine lumen is usually of less significance due to the relatively small amount of blood lost from the circulating pool in most cases.

Diagnosis

Clinical parameters of horses experiencing acute blood loss and hemoperitoneum have been reviewed.[10,15] Consistent signs included depression, tachypnea, tachycardia, poor pulse quality, pale mucous membranes, prolonged capillary refill time, cool extremities and abdominal discomfort. Signs occurring less often included abdominal distention, sweating, ataxia, and a mass in the broad ligament palpated *per rectum*.

Clinical pathology findings include anemia, neutrophilia, lymphopenia, thrombocytopenia, hypoproteinemia, hypocalcemia, and azotemia. Measurements of hemostasis (prothrombin time, parital thormoboplastin time, template bleeding time) are usually normal.

The diagnosis of hemoperitoneum is confirmed by transabdominal ultrasonography and abdominocentesis. Lesions at necropsy may include a ruptured miduterine artery or ruptured broad ligament hematoma. On occasion, the source of hemorrhage may be the iliac vessels.

Rectal examination is controversial, with the need to make a diagnosis balanced by the concern of worsening hemorrhage. There is no evidence that rectal examination of mares suffering prepartum hemorrhage adversely affects outcome.[12] Rectal examination may not aid diagnosis, as the hemorrhage may dissect between tissue planes and not form a discrete, palpable mass in the broad ligament. Careful transrectal ultrasonography may greatly aid diagnosis due to the ability to detect non-palpable lesions.

Treatment

Due to the sporadic nature of the condition, treatment modalities are derived by extrapolation from studies on humans, laboratory species and anesthesia studies in the horse. A number of treatments have been recommended, with both scientific and anecdotal backing for those in common usage:

□*-aminocaproic acid.* The effects of □ (epsilon) aminocaproic acid (EACA) on coagulation and fibrinolysis in healthy horses have been reviewed.[16,17]. Partial thromoplasin time (PTT) was found to be significantly decreased and $\square_2$-antiplasmin activity was significantly higher. Fibrinogen was significantly lower than baseline. Bolus dosage is more practical in field situations; however a recent review[17] established an efficacious constant rate infusion protocol.

The procoagulant EACA is a synthetic anti-fibrinolytic amino acid. Similar agents are widely used in human medicine to arrest hemorrhage. The lysine binding sites of plasminogen become saturated with EACA which displaces plasminogen from the fibrin surface stabilizing the hemostatic plug.[18]

Naloxone. Naloxone is anecdotally reported and widely used in the treatment of postpartum mare hemorrhage.[3] Experimental evidence shows that endorphins released by stress act on opiate receptors to depress cardiovascular function during hemorrhagic shock.[19] Naloxone acts as a μ-opioid receptor competitive antagonist and also has (lesser) antagonist action at the κ- and δ-opioid receptors. The hemodynamic effects of blood loss were

shown to be ameliorated by intravenous administration of naloxone as evidenced by an increase in arterial pressure, left ventricular function and cardiac output.[20,21] Regional blood flow differences were noted in the dog, with naloxone improving circulation to the myocardium, intestine, liver and adrenal.[22] In the horse, naloxone (0.20 mg/kg iv) immediately following acute hemorrhage was found to counter the increase seen in heart rate.[23] However, this would result in a considerably higher dose than that commonly in usage (8 mg) for the hemorrhaging mare. At available concentrations (0.4 mg/ml) a volume of 250ml would have to be infused to achieve the higher dose rate shown to be effective in acute hemorrhage situations.

Dexamethasone or other corticosteroids. The beneficial effects of dexamethasone administration during hemorrhagic shock has been shown in dogs.[24,25]. Increased mean arterial pressure was noted, as was improved blood flow to the pulmonary, gastrointestinal and renal circulations. Furthermore, less cell damage was evident as shown by decreased plasma enzyme elevations referable to damaged tissues.

Formalin. Formalin activation of platelet function during fixation *in vitro* has been reported.[26] The procoagulant properties of formalin (aqueous formaldehyde) in the horse have been critically reviewed.[27] In spite of a reported decrease in clotting and bleeding time in goats,[28] administration was shown to have no effect on primary or secondary hemostasis in normal or aspirin-treated horses.[27] Behavioral effects, tachycardia, lacrimation, salivation and muscle fasciculations were seen at higher doses. Despite no effect on coagulation seen in that study, the usage of intravenous formalin for its purported hemostatic properties is widely practiced in equine medicine.

Yunnan baiyao. Yunnan Baiyao (or Yunnan Paiyao, literally white medicine from Yunnan) is a hemostatic powder of largely unknown constituents. Purported uses include hemostasis, relief of pain, diminishment of swellings and the improvement of circulation to the tissues. The mechanism of action is unknown. Experimentally, template bleeding time of halothane anesthetized ponies was decreased when compared to baseline values following the administration of yunnan baiyao 4 h prior to and immediately preceding induction of anesthesia.[29] Activated clotting time was not affected in this study. Anecdotal reports indicate widespread usage by equine practitioners with reported favorable results.

Acepromazine. The pharmacokinetics and pharmacodynamics of intravenous acepromazine have been extensively reviewed in the horse.[30,31] Use in the hemorrhaging mare is controversial as concerns are held for the potential exacerbation of hypovolemia. However, use allows the hypotensive restoration of adequate circulatory blood volume with a diminished chance of dislodging the hemostatic plug.

Butorphanol. Butorphanol tartrate is widely used for control of pain and chemical restraint in the hemorrhaging mare. Concurrent judicious use of the α_2 adrenergic agonists (xylazine, detomidine) further aids in control of anxiety. Butorphanol is a partial agonist/antagonist at the μ opioid receptor and an agonist at the κ opioid receptor. Therefore, potential for antagonism exists with the concurrent usage of butorphanol and naloxone.

Blood transfusion. Whole blood is the fluid of choice in cases of hemorrhagic shock. However, this is often not available; therefore isotonic polyionic solutions are administered to maintain circulating volume, with consideration of their relatively short time within the vascular space. When blood is lost from the intravascular compartment, central venous pressure decreases and blood lactate concentration increases significantly when compared with baseline values in the healthy horse.[32,33] Surprisingly, heart rate and venous blood gas analysis do not change significantly in the initial period. Therefore, blood lactate concentration is a useful measure of hypovolemia in horses in situations of acute loss before other parameters become abnormal. Also, it may be useful to indicate the need for blood transfusion and monitor responses of horses when whole blood is administered.[32]

Plasma. The administration of plasma is widely practiced for the provision of clotting factors and oncotic support to the hemorrhaging mare. This is useful in situations where anemia is severe once the mare is stabilized and whole blood is not available. In an emergency situation, this is less practical as plasma needs to be administered slowly and the benefit of administration will not be realized in a clinically relevant time frame. Volumes in common usage (1 L) are unlikely to measurably affect oncotic pressure and hemodynamic performance.

Hypertonic saline. In situations of acute blood loss, the restorative fluid used is of lesser importance as long as an appropriate volume is given.[34] In human medicine, considerable interest has been shown in the use of hypertonic saline dextran (HSD) in situations where significant hemorrhage has occurred.[35] Controversy still surrounds the use of hypertonic solutions for rapid restoration of intravascular volume.[36] In the hemorrhaging mare, use of hypertonic saline is widely practice but similarly controversial due to the possibility of rapid plasma volume expansion causing a deleterious rapid spike in blood pressure.

Hetastarch. Hetastarch, 6% hydroxyethyl starch solution, is an artificial colloid used as a plasma volume expander. It has oncotic activity only and is not a blood or plasma substitute. Hetastarch is elimintated over a prolonged period by the kidneys. In situations where rapid plasma volume expansion is needed, hetastarch can be given as a series of rapid bolus doses in contrast to plasma which must be slowly administered. For this reason it offers an attractive way to rapidly ameliorate the effects of acute blood loss. However, one retrospective study

suggests that intraoperative use of hetastarch in human cardiac surgery may increase bleeding and subsequent blood transfusion requirements.[37]

Polyionic replacement fluids. Volume restoration by polyionic fluids is widely practiced in the hemorrhaging mare. These fluids rapidly leave the vascular space (30 minutes) and do not provide a long term solution to hypovolemia, but instead provide a rapid transient means to combat blood loss. When used in conjunction with colloids (plasma and hetastarch) or hypertonic saline a more prolonged effect can be expected. Care must be taken to avoid overzealous plasma volume expansion to preserve the hemostatic plug.

Lidocaine infusion. The historical use of lidocaine as a systemically administered analgesic for intractable human pain has been reported.[38] Analgesic effects in horses have only been relatively recently reported.[39] When practical, a constant rate infusion of lidocaine is an excellent analgesic for the hemorrhaging mare, especially if hemoperitoneum is present. An appropriate dosage regimen is an initial lidocaine loading dose (1.3 mg/kg iv) as a slow bolus, followed by a constant rate infusion (0.05 mg/kg/min iv), preferably using a fluid pump however this is not essential.

Management

Although controversy exists as to the utility of various therapeutic agents, ensuring the mare is as calm as possible and not exposed to undue stress is widely agreed upon. Care should be taken during restraint to not excessively stress the mare by using a combination of physical and chemical restraint.

Broad spectrum antimicrobial therapy is indicated to prevent the establishment of bacterial overgrowth in any hematomas or stagnant pools of blood post hemorrhage. Anti-inflammatory therapy should be maintained following the initial insult to minimize pain and distress, which may lead to increased blood pressure and restarting of hemorrhage.

Fluid therapy should be approached with caution and closely monitored. Rapid plasma volume expansion can lead to hemodilution, loss of the hemostatic plug and restarting of hemorrhage. This must be weighed against the necessity of restoration of an adequate circulating volume in the hypovolemic mare. The mare will succumb to hypovolemia not anemia in the acute phase of blood loss.[34] The signs of hemorrhage and hypovolemia are well known: visible distress or colic signs, muscle fasciculations, sweating along the flanks, flehmen, elevated heart and respiratory rates, and palor of the mucous membranes. Should these signs return during fluid restoration, an immediate decrease in the rate of admission should be considered. In the healthy horse, where potential for hemorrhage is not present, one-half of the calculated fluid deficit can be administered rapidly, with the remainder of the deficit given over the ensuing 24 h. However, in the mare affected by peripartum hemorrhage, this initial rapid high volume administration is not possible.

A representative treatment plan for the author follows. Subsequent to the diagnosis of hemorrhage, an intravenous catheter is placed and an initial 5 L bolus of polyionic fluids containing 20 g □-aminocaproic acid as a hemostatic agent is given over 30 min. Concurrent with this, acepromazine is administered intramuscularly to allow hypotensive circulating volume restoration and to act as a mild calming agent. Unless the mare is showing obvious signs of cardiac compromise, naloxone is not administered. Broad spectrum antimicrobial coverage is initiated (K penicillin and gentamicin, alternatively trimethoprim-sulfamethoxazole) for a minimum of five d. Analgesia and anti-inflammatory therapy is provided by flunixin meglumine. When this is insufficient, a constant rate infusion of lidocaine is given until 48 h following the last noted abdominal pain. Hemostatic therapy is continued for 2 to 3 d, allowing ultrasonographic evidence of the cessation of hemorrhage and stabilization of the hemorrhage site (if visible or palpable) to be noted. The author avoids the use of oxytocin in post partum hemorrhage mares, and is judicious with rectal evaluation. Uterine lavage, when attempted after 2 to 3 d, involves the establishment of a siphon and avoids distension of the uterus whenever possible. Undue stress from any source is avoided. This is especially important with mares protective of the foal, where procedures involving the foal are minimized and absences will be avoided if at all possible.

An appropriate fluid plan following stabilization of the hypovolemic mare is to provide a maintenance rate of polyionic intravenous fluids (2 ml/kg/h) until water intake is deemed sufficient. A PCV that is low but stable is acceptable. The author considers a PCV of 15% that is stable acceptable, however a blood cross match is initiated at this point. The PCV will begin to slowly rise (1 to 2% daily) once hemorrhage ceases and the bone marrow responds. Resorption of peritoneal blood (if present) aids in this increase.

Should the mare reach a PCV of 12% and continue to decrease, up to 20% of the mare's circulating volume should be replaced with whole blood over 2 to 3 h by transfusion from a compatible donor. In this case, it should be expected that the PCV will begin to slowly decrease again over a period of 2 to 3 d as the transfused red blood cells are removed from circulation.

Useful drug dosages for postpartum mares are summarized in Table 1.

The Foal

Peripartum risk factors

Peripartum factors affecting the neonatal foal may be divided into three broad categories.

Maternal health. Systemic illness with fever, gastrointestinal compromise (potential for endotoxemia), and surgical manipulation are deleterious to the fetus. Nutritional status also affects fetal health.[1]

Reproductive conditions of the mare. History of previous neonatal compromise, known placental pathology (infection, thickening, separation), abnormalities of the birth canal, vulvar discharge, and loss of colostrum prepartum alert the clinician to potential neonatal difficulties.

Parturient events. The neonate can be affected by an abnormal gestation length, prolonged labor, dystocia, premature placental separation, and premature rupture of the umbilical cord. Meconium aspiration leads to hypoxic injury and pulmonary disease, with presence of meconium in the amniotic fluid or amnion sometimes the only indication of this event.

Resuscitation of the compromised neonate

Prior preparation in anticipation of an emergency is paramount to success. A readily available collection of necessary equipment and drugs, kept within an easily accessible and portable container aids in achieving a successful outcome. A list of drug dose rates useful in resuscitation situations should be kept within the drug kit (Table 2).

Preparation of the foal for resuscitation includes drying and generally stimulating the neonate, and clearance of respiratory and oral secretions by suctioning to maximize the airway. It is convenient and safest to position the foal in lateral recumbency with any rib fractures down.

ABCDE of resuscitation

Establishing a clear and consistent protocol for resuscitation of the neonate is important when seeking to avoid delays in action during emergency situations (Table 3). The order of activities can easily be remembered by the ABCDE protocol. Techniques, drug dosages and break points determining changes in action may vary between individual clinicians due to preference and case experience. The aim of establishing a regular and sustainable cardiac and respiratory rhythm however is common to all protocols.

Indications for referral

The normal newborn foal displays a fairly predictable progression from the time of delivery to the onset of appropriate foal behavior and physiology (Table 4). Significant deviation from these benchmarks strongly suggests that referral for advanced care should be considered.

Evaluation of the *at risk* foal includes a physical examination and consideration of the gestational history and laboratory values if available (Table 5). Although the foal may appear normal initially, rapid deterioration is possible and subtle deviations in physical findings and blood values may be the only indication of impending trouble.

Stabilization of the foal and preparation for referral

If the foal is showing signs of distress it may be prudent to transport the foal as soon as possible even if separately from the mare. Send colostrum from the mare for later administration and the placenta for examination if available.

If hypothermic, ensure the foal remains warm. Use blankets, insulated foal covers, or provide external sources of heat such as warmed fluid bags. Avoid excessively warming the hypovolemic foal as increasing circulation at the periphery can lead to profound falls in blood pressure.

If breathing difficulties are present, place an intranasal oxygen cannula. Insert the tip to the level of the eye socket. Portable oxygen tanks can be set to provide 5L oxygen flow per minute.

Fluids should be administered if dehydration or hypovolemia are present. Half the calculated deficit (deficit in liters = bodyweight in kg x % dehydration) can be given rapidly prior to referral. Maintenance fluid rates for foals are higher than the adult horse, being 5% to 10% of body weight daily, i.e., 2 to 4 ml/kg/hr. When calculating the required maintenance rate, consider all sources of fluid intake for the foal to avoid over hydrating the compromised foal. This is especially important if the foal is recumbent as exceeding 10% of bodyweight can promote pulmonary edema formation. Glucose supplementation (2.5% or 5% dextrose in polyionic fluids) can be given if blood glucose levels are low, however rapid rehydration of the foal should always use non-glucose containing fluids. Care must be exercised with the addition of glucose to fluids as over supplementation results in hyperinsulinemia and subsequent worsening of hypoglycemia.

Placement of an indwelling feeding tube will aid in administration of colostrum as well as provide a vehicle for continued feeding of the foal (if appropriate) during transport to the referral facility should this be distant from the farm. Ensure the foal is fed standing (if able to rise) or only when in sternal recumbency. Reflux of gastric content is still possible with a correctly placed nasogastric tube. Avoid overfeeding the sick foal: a useful rule of

thumb is to feed 10% of the bodyweight of the foal as milk over a 24 h period. Divide this amount into 12 equal feeds at two h intervals. Should colic or nasogastric reflux occur post feeding, discontinue immediately.

Antimicrobial therapy should not be delayed in the foal suspected of sepsis (Table 2). Collection of a blood culture using aseptic technique before administration of antimicrobials is desirable to improve chances of yielding the causative infectious agent. This can be shipped with the foal for bacteriologic examination and antimicrobial sensitivity analysis.

Anti-inflammatory treatments are also indicated in the foal that has sustained physical trauma or is febrile. Lipid derived inflammatory mediators are important in the pathophysiology of hypoxic ischemic encephalopathy (HIE) suggesting prompt use of non-steroidal anti-inflammatory drugs (NSAIDs) and anti-oxidant therapies is warranted. The NSAIDs vary in their potential for gastric mucosal and renal toxicity, especially if used in the dehydrated patient; therefore care must be exercised in selection for the compromised neonate.

If neurological dysfunction is present, control of cerebral edema and seizure activity (if present) are indicated. Cerebral edema results from any traumatic, ischemic or hypoxic insult to the neonate. The onset of signs of neurological dysfunction is usually delayed, becoming apparent after a 24 to 48 h period of apparently normal development. Often the only suggestions of impending problems are the gestational history, parturient events, and subtle early neonatal behavioral abnormalities. Should seizure activity ensue, control of seizure activity in the first instance is necessary to avoid rapid exhaustion of the foal and secondary injuries predisposing to bacterial sepsis. Placement of leg wraps and provision of padding adjacent to the foal will minimize trauma.

If the foal is sufficiently medically stabilized and the owner is compliant, transport to a referral facility is possible with the following considerations:

Timeliness. Nothing is worse than a referral too late. Increased costs of treatment to the owner coupled with a decreased prognosis result in a loss to us all as a profession.

Owner financial resources. Hospitalization will be expensive. Complications are provided at no extra cost. Continuous nursing care is expensive but imperative.

Ensure secure vascular access; if you place an intravenous catheter ensure that it will remain in place with all attachments. This may be replaced with a longer-term catheter in hospital.

History. Where possible, a written account encompassing treatment to the time of arrival at the hospital. If the responsible person is not coming with the foal, encourage the client to bring your billing/record sheets.

Up to date blood work is imperative. Blood collected before referral will likely be repeated, to establish both a baseline and to gauge response to previous treatments.

Mare compliance. Is it necessary for the mare to accompany the foal? If so, will she adjust to a hospital setting?

Summary

Peripartum hemorrhage in the mare may become a life-threatening emergency depending on the structures involved and the extent of blood loss. Treatment of the mare centers on promoting hemostasis and restoring circulating blood volume in a fashion that does not excessively raise blood pressure and risk restarting hemorrhage. Many established treatments are controversial. The neonatal foal may require resuscitation following delivery as the result of gestational or parturient events. Although many conditions can be managed on the farm, a thorough clinical exam should be performed to identify those foals in need of specialist intervention. Appropriate stabilization and preparation for transport and referral increase the probability of success.

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Table 1: Useful drug dosages for the postpartum mare

Sedation:
Care should always be exercised in the administration of sedation to the pregnant mare. Most agents cause hypotension which will exacerbate the negative effects of hypovolemia.
Xylazine: 0.25 to 1 mg/kg iv or im. Potent hypotensive effects.
Detomidine: 0.01 to 0.02 mg/kg iv or im. Less hypotensive potential than xylazine.
Butorphanol: 0.01 to 0.02 mg/kg iv or im. Give as needed, may be repeated every 8 to 12 h. Effects will be attenuated where naloxone is administered concurrently.
Acepromazine: 0.02 mg/kg im q 12h. Potent hypotensive agent, use with caution.

Analgesia:
Flunixin: 1 mg/kg iv q 12h
Lidocaine: load with 1.3 mg/kg slow iv bolus of lidocaine 20% followed by 0.05 mg/kg/min iv as a constant rate infusion. May be conveniently made by adding 450 ml lidocaine 20% to a 3 L fluid bag and administering at 1 ml/kg/h.

Hemostatic agents:
□-aminocaproic acid: 40 mg/kg iv load then 20 mg/kg iv q 6-8h. Administer in 1 L fluids over 15-30 min. Five or more doses may be required if hemorrhage ongoing.
Yunnan baiyao: 8 mg/kg po q 6h. Administer 16 x 0.25g capsules in water as paste q 6h. Duration of treatment is highly variable, being 2 to 4 d.

Circulatory support:
Naloxone: 0.01 to 0.02 mg/kg iv. Administered in initial treatment phase, may be repeated.
Polyionic fluids: 2 ml/kg/h iv (maintenance rate). Continued throughout the period when fluid intake by other means deemed inadequate.
Hypertonic saline: 2 to 4 ml/kg iv. Controversial due to potential for a rapid rise in blood pressure. Given during the initial fluid resuscitation period.
Hetastarch: 6 to 10 ml/kg iv. Administered during the initial fluid volume restoration period only.

Table 2: Useful drug dosages for the foal.

Antimicrobials:
Duration of treatment is governed by clinical response. A minimum of five d should be administered in the absence of adverse reactions.
K penicillin: 40000 to 50000 units/kg iv q 6h
Ceftiofur sodium: 2 mg/kg im q 12h or 5 to 10 mg/kg iv q 6-12h
Amikacin: 25 mg/kg iv q 24h
Trimethoprim-sulfamethoxazole: 30 mg/kg po bid

Seizure control and metabolic support:
Diazepam 0.05 to 0.4 mg/kg iv. Short-acting control following acute onset of seizure activity. May repeat 2 to 3 times as required to establish control.
Phenobarbital 4 to 10 mg/kg iv. Long-acting control useful when initial agent fails. May be repeated at 12 h intervals for continued control, or change to oral phenobarbital at 4 mg/kg po q 12h.
Thiamine 10 mg/kg iv q 24h. Cerebral metabolic support. Useful in initial stages of hypoxic insult.

Control cerebral edema and inflammation:
Hypertonic saline 7 ml/kg iv as 3% solution. Shown to aid in control of cerebral edema. Useful in initial period following insult.
Mannitol 1 mg/kg iv as 20% solution. May repeat at 12 h intervals until appropriate mentation returns, often 2 to 3 d sufficient.
Flunixin meglumine: 1 mg/kg iv q 12h. Continue while clinical signs evident.
Ketoprofen: 2 mg/kg iv q 24h. As for flunixin. Less ulcerogenic potential.

Control oxidative damage:
Vitamin C 100 mg/kg iv q 24h. Useful in the initial stages following onset of cerebral compromise. Three d treatment or more may be required and this is safely administered.
Vitamin E 20 iu/kg sc q 24h. Requires prolonged administration to reach therapeutic levels in central nervous system.
Dimethyl sulfoxide 1 g/kg iv q 12h as 10% solution. Useful during period when ongoing damage is suspected to be occurring, often up to three d post insult.

Respiratory stimulant
Caffeine 10 mg/kg po load, then 2.5 mg/kg po q 6h. Continue until appropriate respiratory pattern is established. May cause hyperactivity and lower the seizure threshold in some foals.

Table 3: Resuscitation protocol for the compromised neonate.

Airway

- Intubate by nasotracheal route with largest practical endotracheal tube
- Extend neck and twist as arytenoids reached to ease passing
- Size **8 to 10 mm** suitable for average foals
- Pass tube to nares to minimize dead space
- Compress chest and palpate esophagus to ensure correct placement

Breathing

- Respiratory arrest usually precedes cardiac arrest in the neonatal foal
- Establish rate of **8 to 10** breaths/minute with 1 second inspiration period
- Use 100% oxygen if available, however room air is acceptable
- The use of a self-inflating resuscitation bag with a pressure limiting valve (**Ambu**), or any other similar delivery device, will aid ventilation and avoid excessive inflation pressure
- **Doxapram** (controversial) at **0.5 mg/kg** iv

Circulation

- Thoracic compressions if **HR less than 60**, especially if less than 60 and not increasing
- Minimize interruptions (no longer than 10 seconds)
- Rapid compressions (aim for **100/minute**)
- Establish vascular access if response is not immediately favorable

Drugs

- Usage and rationale based on experience and extrapolation
- Heart rate **less than 60 and not increasing**
- Continue compressions to distribute drugs
- **Epinephrine** at **0.02 mg/kg** (0.5 to 1 ml of 1:1000 per 50 kg foal). Intratracheal dose 5 to 10x this, however absorption is poor
- Repeat every 3 to 5 minutes until response is noted
- Administer **10 ml/kg** of a balanced electrolyte solution. A 2 to 4 ml/kg hetastarch bolus may be useful to rapidly expand circulating volume
- Avoid inducing hyperglycemia with dextrose containing solutions as resulting hyperinsulinemia is depressive
- **Dobutamine** at **3 to 40** □**g/kg/min** iv is useful to improve pulse pressure and peripheral perfusion

Everything else

- Monitor foal progress
- Pupillary light responses: dilated pupils indicate lack of cerebral perfusion
- Stop when HR above 60 (pause 10 seconds max)
- Spontaneous breathing (pause 30 seconds)
- End point determination

Table 4: Times of importance to the neonatal foal.

- Sternal Recumbency: the foal should right itself and be able to remain sternal within 5 to 10 minutes of birth.
- Standing: within 60 minutes (range of 15 to 165 minutes). Compromised neonates tend to remain recumbent longer, further exposing themselves to pathogens.
- Suckle reflex: usually develops within 20 minutes of birth, although may be much sooner.
- Suckling: the foal should suckle the mare within 2 h (range 35 minutes to 7 h).
- Urination: first urination occurs at 6 h for colts, 10 h for fillies.
- Urine production: approximately 6 mL/kg/hr. Decreases may result from decreased fluid intake, increased losses or compromises in renal function. Obstruction or disruption due to rupture and uroperitoneum are possible in the compromised neonate, or one which sustained trauma during parturition.
- Defecation: foals display abdominal straining within the first few h after of birth, and pass meconium completely within 24 h. Colostrum stimulates GI motility. Any interference with GI motility will prolong passage of meconium increasing the likelihood of impaction.

Table 5: Examination of the neonatal foal.

- Rectal temperature
  Appropriate neonatal range 99 °F to 101.5 °F. Neonatal foals are unable to regulate body temperature to the same degree as older foals.

- Cardiovascular system
  Appropriate neonatal heart rate (HR) range is between 70 to 120 beats per minute. HR is highly labile, however rate and rhythm is regular. Pulses are synchronous with the heart beat and easily palpable. Deviations may indicate arrhythmia (electrolyte abnormality, congenital cardiac anomaly). A murmur associated with a PDA can occur for the first few days of life.

- Respiratory system
  Within the first hour of life, the respiratory rate (RR) of a normal foal can rise up to 80 breaths per minute. The RR decreases progressively over the next few days, with a subsequent range of 30 to 40 breaths per minute. Increased RR may indicate compromised pulmonary function, pain, excitement or fever. The magnitude of the thoracic excursion is indicative of the respiratory effort. Any decrease may indicate fatigue. Nostril flaring may be the only indication of increased respiratory effort.

- Musculoskeletal system
  Joint distension: indicative of sepsis, coagulopathy (hemarthrosis), trauma.

- Integument
  Decubital ulcers indicate trauma, unseen seizure activity or the occurrence of prolonged recumbency. Pitting edema suggests hypoproteinemia or cardiac dysfunction. Icterus suggests sepsis, neonatal isoerythrolysis, or hepatic disease. Hemorrhage on the mucous membranes suggests sepsis, coagulopathy or direct trauma.

- GI function
  Any occurrence of colic has the potential to indicate a life-threatening episode and should be thoroughly investigated.

- Urinary function
  Acute renal failure may occur as the result of decreased *in utero* blood supply or be iatrogenic from nephrotoxic drug usage. Uroperitoneum results from a ruptured bladder, ruptured urachus or torn ureter.

- Ocular examination
  Hyperemia of the sclera indicates birth trauma, sepsis, or coagulopathy. Entropion results from weight loss or dehydration leading to enophthalmia. Corneal opacity is the result of ulceration, a depressed blink response, lack of tear production, or exposure keratitis in the depressed neonate. Anterior chamber: can reflect systemic inflammation and sepsis. Fibrin deposition (aqueous flare) and hypopyon may result.

- Mentation and nervous function
  Hypoxic ischemic encephalopathy causes loss of affinity for the mare, generalized depression and a lack of vigorous suckling. Low level seizure activity may appear as muscle fasciculations, chewing fits, or unexplained cutaneous trauma.
  The menace response is absent from the neonate and is not an indication of vision. This reflex will take between 4 to 14 d to develop.

HEMATOLOGY

- Normal neonatal range (birth to 1 week): RBC range from 7.4 to 11.4 million cells/uL. WBC range from 4.9 to 13.6 thousand cells/uL.
- Neutrophils (N): approximately 5500 cells/□L are present at birth with this increasing to 8000/□L within the first 12 h of life.
- Lymphocytes (L): may decrease to approximately 1400 cells/□L within a few hours of birth, thereafter they increase to approximately 5000 cells/□L by 3 months of age. A transient decrease to below 1000 cells/□L may occur in some normal foals, but this may also indicate infection or immune compromise.

SERUM CHEMISTRY

In the absence of established foal parameters in many laboratories, normal adult equine values are often used for assessment of neonatal foal health.

- Serum electrolyte concentrations are maintained within a narrow range and do not differ substantially from established adult values.
- Glucose is elevated compared to adults due to frequent suckling. Hypoglycemia is cause for concern as this may indicate of sepsis or decreased feeding activity.
- Plasma protein levels at birth vary considerably between foals.
- Serum creatinine and BUN are unreliable indicators of newborn foal renal performance as the placenta is primarily responsible for elimination of waste products; therefore an increase reflects placental dysfunction. However, BUN and creatinine may be initially elevated decreasing to adult levels by 3 to 5 d.
- Total bilirubin may be elevated as mild icterus is common. Consider sepsis, isoerythrolysis or hepatic disease.
- Creatine kinase is raised by muscle trauma from delivery.
- Alkaline phophatase is elevated due to rapid growth in the neonate.
- GGT elevations may be of colostral origin.
- Fibrinogen is uniformly low in the healthy neonate (up to 200mg/dL). Elevated levels indicate *in utero* challenge with prenatal response.
- IgG concentration: there is a strong association between the occurrence of sepsis and an immunoglobulin concentration less than 400mg/dL. Catabolism of immunoglobulins may occur in the compromised neonate.

Equine metabolic syndrome and Cushing's disease: Possible role in infertility in the horse

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Abstract

Over the last several years, there has been a clear increase in the geriatric horse population. Some of those mares are expected to become donors for embryo transfer or to become pregnant, forgetting the consequences of a long life expectancy. For example; illnesses and problems that commonly affect the aged horse - such as Cushing's disease (CD) and equine metabolic syndrome (MS) - have become more prevalent. These metabolic conditions share traits, e.g., insulin resistance (IR), often in conjunction with obesity, which have been associated with the development of abnormal reproductive function and laminitis in the horse. A large number of obese and IR mares continue to cycle during winter, have longer inter-estrus intervals, prolonged luteal phases and a higher incidence of anovulatory follicles. Diagnosis of MS or CD requires a good clinical assessment in combination with the proper laboratory testing. Once identified, MS should be managed with a diet poor in carbohydrates and an exercise program that stresses a moderate body condition. Treatment for CD comprises appropriate health care and the administration of pergolide, cyproheptadine or a combination. Even though a link between infertility and these metabolic conditions has been established, there is a clear need for further investigation.

Keywords: Cushing's disease, metabolic syndrome, insulin resistance, infertility, mare

Introduction

Several endocrine and metabolic conditions had been empirically linked to infertility in the mare including hypothyroidism, metabolic syndrome (MS-insulin resistance) and Cushing's disease (CD) or pituitary pars intermedia disease (PPID). The mechanisms by which these conditions lead to infertility are not completely understood. These metabolic aberrations are gaining attention by veterinary scientists and equine nutritionists as the population of geriatric, obese horses in the USA is increasing and owners are requesting additional care for affected horses. This review discusses the pathophysiology of MS and CD, how they may adversely affect fertility, diagnostics and recommended therapies.

During the fall months and in preparation for winter, the horse begins to store energy in the form of body fat. The process is controlled by the hypothalamic-pituitary axis as it induces an increased secretion of pro-opiomelanocortin peptides[1] and results in an increase in appetite and adipogenesis in conjunction with the development of a thick hair coat. This "survival mechanism", disappears by the end of winter in healthy horses but not in horses with MS or CD. Metabolic synrrome and, to a certain degree CD, are associated with the acquisition of excessive fat and, more importantly, the persistence of body fat and its negative consequences on the mare's general heath and reproduction.[2] Clinical findings that should make one suspicious of MS or CD include obesity in older horses that do not shed their winter coats completely or do so much later in the spring than the remainder of the herd, abnormal estrous cycles, anovulatory follicles and repeated bouts of endometritis. A number of tests are available for diagnosing MS or CD. However, there is no single "gold standard".

Equine metabolic syndrome

Equine MS is closely associated with two disorders: obesity and insulin resistance (IR or glucose intolerance). Horses affected with MS also have a predisposition to developing laminitis and mares may exhibit abnormal estrous cycles.[3] The obesity is evident as subcutaneous regional adiposity ("cresty neck", tailhead, shoulders and prepuce in geldings) and affected horses tend to be genetically predisposed requiring fewer calories to keep normal body weight ("easy keepers"). Not all obese horses have IR even though obesity increases the horse's risk of developing the disease. Some breeds appear to be more commonly affected than others, including ponies, Quarter Horses, Morgans, Arabians and Saddlebreds. There is no gender bias.[4]

Pathophysiology: When foods rich in carbohydrates are ingested, they are broken down into glucose. This is followed by a physiological hyperglycemia which triggers the release of insulin from the pancreas. Insulin promotes glucose uptake by cells. Once in the cell, glucose is either used immediately or it is stored as glycogen or fat. The amount of insulin required for inducing glucose uptake into cells is tightly regulated and is referred to as insulin sensitivity. If a horse develops IR, there is a reduction in the insulin driven uptake of glucose by the skeletal and adipose tissue creating hyperglycemia.[2,4] This, in turn, generates hyperinsulinemia in an effort by the pancreas to compensate for the insensitivity.

Little is known about the factors predisposing to IR; however, obesity appears to be the recurrent trait in horses. It has been suggested that certain adipocites behave as endocrine tissue releasing cortisol, pro-inflammatory cytokines and leptin, which may interfere with glucose uptake by the cells.[2] Age and diet appear to be important contributing factors to the development of IR. Ageing is believed to decrease sensitivity to insulin.

Obesity in horses and especially in ponies appears to be a risk factor for mild laminitis. The simplest explanation is excessive weight at the hoof–lamellar interface.[4] The fact that ponies are at higher risk even though they bear less weight, suggests the genetic predisposition of this breed to MS and IR.[2,4,5] Glucose is essential for the health and strength of the hoof-lamellar interface. IR creates cell-glucose starvation which may increase the risk of laminitis more than obesity per se.

Diagnosis

Characteristics that are common in horses with MS or IR include increased age (8 to 20 years old), obesity, distinctive distribution of body fat (neck, shoulders, rump), "easy keepers" that have difficulty losing weight, a history, presence or predisposition for laminitis, abnormal estrous cycles and decreased pregnancy rates. Resting levels of insulin are good indicators of IR since compensatory hyperinsulinemia is a common finding. Plasma samples should be taken early in the morning after an overnight fast. Horses should be removed from pasture and only fed course roughage overnight. Roughage should be available as some horses will become increasingly agitated if no food is available. Resting insulin concentrations > 20 μU/mL are suggestive of insulin resistance and > 30 μU/mL are considered diagnostic.[4,6] Even though resting levels of insulin are good markers, they are not helpful in mild or early IR where the rise of insulin is too small to reach or exceed the reference value. Reference values differ among laboratories and should be taken into consideration when interpreting results (insulin >30 μU/mL at University of Tennessee vs. >43 μU/mL at Michigan State University).[6]

If one is presented a horse with physical findings consistent with MS or CD that has a normal resting plasma insulin level, a combined glucose-insulin test should be performed.[6] This test consists of collecting a baseline glucose blood sample before infusing 150 mg/kg of 50% dextrose solution. Immediately after the dextrose infusion, insulin is administered intravenously at 0.10 U/kg. Serum samples are collected at 1, 5, 25, 35, 45, 60, 75, 90, 105, 120, 135 and 150 min. However for practical purposes, the test can be limited to 60 minutes. A horse is considered to have insulin resistance if blood glucose levels remain above baseline for more than 45 min.[6] Stress should be avoided during the test since it can cause transient IR. Horses should be kept quiet, allowed to graze and the jugular catheter should be placed the night before for blood collection.

Metabolic syndrome and CD share a number of clinical signs including abnormal distribution of fat, hyperinsulinemia and insulin resistance, predisposition to laminitis and infertility. Cushing's disease can be differentiated from MS by performing a dexamethasone suppression test (DST; see PPID), as this test will be positive only for PPID. Indeed, MS has been described as the presence of insulin resistance without detectable presence of PPID.[7]

Treatment

Treatment is directed at lowering body fat and consequently insulin levels to <30 μU/mL. Horses should be placed on a low carbohydrate diet to reduce caloric intake and partake in a consistent exercise program to increase energy outflow and glucose uptake. Grain should be removed from the diet (alone or mixed with molasses) and access to pasture should be limited.

Medical treatment with levothyroxine sodium (Thyro L; Vet-A-Mix, Shenandoah, IA, USA) is warranted if weight loss occurs too slowly or hyperinsulinemia persists after reaching the desirable body weight.[4] Levothyroxine sodium is administered at a dose of 48 mg/day PO for 3 to 6 months to accelerate weight loss in horses, especially those which cannot exercise due to laminitis. Persistent hyperinsulinemia/hyperglycemia is treated with a combination of levothyroxine sodium (24 mg/day PO) and a biguanide (metformin; 15 mg/kg PO BID). The mechanism of action is not known but it appears to inhibit gluconeogenesis and increases glucose uptake.[4] Its safety in the horse has not been established. In addition, administration of chromium at a dose of 5 to 25 mg/day may improve insulin efficacy in transporting glucose into the cells.[4,7]

Cushing's disease or pituitary pars intermedia dysfunction

Cushing's disease or PPID is a relatively prevalent condition in horses due to an increase in the geriatric population.[8,9] Onset of clinical signs usually occurs around 18 to 23 years of age but horses as young as 7 years have been affected. There is no sex predisposition and all breeds can be affected. Ponies and Morgan horses appear to be at a higher risk.[10] First described by Pallaske 75 years ago, this syndrome is characterized by adrenocortical hyperplasia and excessive glucocorticoid, mineralocorticoid and androgenic steroid secretion in response to a surplus release of adrenocorticotrophic hormone (ACTH).[11] In the horse, there is a hypothalamic or pituitary-

dependant hyperadrenocorticism. The presence of a possible benign adenoma in the pituitary gland or the degeneration of dopaminergic neurons at the hypothalamic level had been held responsible.[6,9,12]

Pathophysiology. The hypothalamus controls secretion of ACTH by the anterior pituitary through the release of corticotrophin releasing hormone (CRH). Neurons secreting CRH are located in the anterior portion of the paraventricular nuclei and their axons terminate in the pituitary median eminence. Corticotrophin releasing hormone stimulates secretion of ACTH, beta-lipotropin (ß-LPH) and ß-endorphins. All three originate from the same precursor called pro-opiomelanocortin (POMC),[9,11] and all have the same secretory dynamics, that is release is increased in response to stress and hypoglycemia and decreased in response to glucocorticoids.
Negative feedback inhibition of ACTH by glucocorticoids is absent in PPID. Therefore, ACTH and POMC related hormones persist despite cortisol elevation.[12]

Chronic exposure to excessive glucocorticoids results in a variety of physical and clinical abnormalities. The most characteristic clinical sign is hirsutism (47 to 100% of affected horses) and the inability to shed the winter coat. It is suggested that hirsutism results from increased melanocyte-stimulating hormone (MSH) released from the pars intermedia or increased production of androgens by the adrenal cortex. In women, hirsutism has been associated with clinical hyper-androgenism.[13] In addition there are changes in weight, unusual fat accumulation at the neck, tail head and over the croup (29 to 67%). Obesity is believed to be the underlying factor for horses with MS to develop PPID at a younger age than average. Chronic obesity is thought to result from a pro-inflammatory and pro-oxidative condition that may accelerate degeneration of dopaminergic neurons.[7] Other characteristics are muscle atrophy (protein catabolism), immunosuppression (and consequent predisposition to retarded wound healing and infections such as endometritis and sole abscesses), polydipsia and polyuria (25%), lethargy, tachypnea, hyperhidrosis (65%) and occasionally, neurologic impairment.[6,14] Laminitis occurs in 50% of advanced cases. One explanation is that excess of corticoids promote laminar vasoconstriction. The presence of IR seems to exacerbate the condition. Broodmares may show persistent lactation (hyperprolactinemia), low grade endometritis (immunosuppression) and anovulatory follicles during the cyclic season.[7,9] In humans, 23% of Cushing's patients with persistent lactation and amenorrhea (due to GnRH inhibition by prolactin) have increased prolactin concentrations. The increase in prolactin is due to excessive production and secretion by corticotroph adenomas or an alteration of the regulation at the dopaminergic neurons.[6,9,12]

The most common biochemical abnormality in PPID is hyperglycemia and hyperinsulinemia/IR. Other findings include increased hepatic enzymes, cholesterol and triglycerides. Horses can also have mild anemia, neutrophilia and lymphopenia.

Diagnosis

Presence of hirsutism had greater diagnostic accuracy than any endocrinological test alone or in combination.[10] Affected horses are usually aged (8 to 20/30 years old), overweight, with the characteristic distribution of body fat (neck, shoulders,), laminitis, and abnormal estrous cycles.[7,8,9,10]

Dynamic endocrinologic diagnostic tests

The dynamic endocrinologic diagnostic tests consist of the overnight dexamethasone suppression test (DST), thyrotropin-releasing hormone (TRH) stimulation test and the cortisol rhythm assay.

DST. In normal horses, administration of dexamethasone (20 mg IM/500 kg) decreases the ACTH release from the pituitary, resulting in a cortisol serum concentration of < 1 µg or 10 ng/dl approximately 19 hours after injection. Conversely, in horses with PPID, serum cortisol concentration does not decrease after dexamethasone administration due to continuous ACTH release. Unfortunately, this gold-standard test loses accuracy (sensitivity and specificity) early in the condition when there is still some feedback from the hypothalamic-pituitary axis, as it appears that loss of suppression by dexamethasone is a late event in the pathogenesis.[10] The DST results are also affected by the season of the year since the pituitary pars intermedia produces more hormones during the fall ("survival mechanism"). One study showed that false-positive overnight DST results were common when horses or ponies were tested in the fall.[1]

TRH stimulation test. Horses with PPID show an increase (30 to 50% from baseline) in serum cortisol concentration 30 to 90 min after TRH administration (0.5 to 1 mg, IV) whereas normal horses do not.[15] Normally, TRH produced by the hypothalamus stimulates TSH release from the pituitary. However, in PPID, exogenous TRH stimulates ACTH and hence, cortisol release. It appears to be due to an up-regulation and expression of TRH receptors in hyperplastic/adenomatous corticotropes in the pituitary pars distalis and intermedia.[10,14] This test is safe to perform in laminitic horses. It has not been critically evaluated in horses early in the disease process.

(Diurnal) Cortisol rhythm assay. This assay is based on the observation that horses with PPID have a loss of the diurnal cortisol rhythm (normally high in morning, low at midnight).[10,16] When performing this test, plasma

cortisol concentration is measured at 8 AM and again at 4 PM. A difference of less than 30% between the morning and afternoon cortisol concentration is considered to be suggestive of PPID. However, this test has not been validated and several factors such as fasting, changes in stabling or laminitis can increase plasma cortisol concentration in the horse.

Combined DST and TRH stimulation test. Administration of dexamethasone before TRH should suppress ACTH release from the pituitary par distalis therefore, any increase in cortisol after TRH administration is attributed to pars intermedia corticotropes. In performing this test, a baseline plasma cortisol concentration is obtained followed by administration of dexamethasone (20 mg, IM/500 kg). Three h after the dexamethasone is given, TRH (0.5 to 1 mg, IV) is administered. Cortisol should be suppressed by the TRH. Plasma cortisol concentrations are measured 30 min and 21 h after administration of TRH (24 h after dexamethasone administration). A plasma cortisol concentration > 1 µg/dl 24 h after administration of dexamethasone or a ≥ 66% increase in cortisol 30 min after TRH administration is considered diagnostic for PPID.[8,10] The test is expensive and may be impractical for the ambulatory clinician.

Accuracy of each test when compared with histological findings is DST/TRH 81%; DST 71%; TRH 71%. Accuracy of hirsutism alone is 86%.

Single sample endocrinologic tests

Two single sample endocrinological tests are available and include measurement of endogenous release of plasma adrenocorticotrophic hormone (ACTH) concentration and insulin concentration.

ACTH concentration test. Measuring a single sample of endogenous plasma ACTH, which is supposedly higher in horses with PPID, is not more accurate than the DST test, does not detect early cases, and it undergoes daily and seasonal variations. A reference value of > 50 pg/ml is considered elevated.[10] Sample handling is intricate. Blood must be collected in cold plastic tubes containing EDTA, as the ACTH can be absorbed onto glass and degraded by proteolytic enzymes in blood or serum. It needs to be centrifuged as soon as possible (within 30 to 60 min) and the plasma transferred immediately to a plastic tube for freezing and shipping (should arrive to the laboratory below 60 ^{0}F). This test is recommended when one is trying to avoid administration of dexamethasone to laminitic animals.

Insulin concentration. Insulin resistance/hyperinsulinemia has been recognized in horses with PPID. Affected horses have increased insulin blood levels attributed to excessive circulating concentrations of cortisol since they have antagonistic metabolic effects. Excessive insulin concentrations vary between laboratories and reference values must always be consulted when interpreting the test. Cutoff point for normal insulin concentration varies from 25 µU/ml to 57 µU/ml[7,10] however, > 30 µU/ml is considered diagnostic of hyperinsulinemia. The accuracy of this test compared to histological findings is 92%. This test has the advantage of a single sample for the diagnosis, however, MS should be ruled out since hyperinsulinemia is also present in this condition.

Douglas has proposed that testing for suspected cases of PPID and MS should start by simply collecting two serum samples 8 to 10 h apart.[7] No grain should be given 4 h before both samples and stress should be avoided since both parameters can result in false positives. Serum samples are collected for measurement of cortisol, insulin and thyroxine (TT4). The absolute values of cortisol are 20 to 90 ng/ml, however, the test specifically looks at a difference of less than 30% between the two samples since that indicates a loss in the diurnal cortisol rhythm which is considered suggestive of PPID. A consistent insulin value > 25 to 30 µU/ml will be indicative of IR.

A typical endocrine profile in a horse with IR and PPID

Sample time	**TT4** **Normal=12.0 ng/ml**	**Insulin** **Normal=<25 µU/ml**	**Cortisol** **Normal 20-90 ng/ml**	**Cortisol** **Normal>30 % difference**
8 to 10 AM	11.9 ng/ml	35 µU/ml	58 ng/ml	11 %
4 to 6 PM	12.2 ng/ml	55 µU/ml	65 ng/ml	

From: Douglas RH. Endocrine assessment and management of insulin resistance and PPID.[7]

Horses suspected of having early signs of PPID ("Pre-Cushingoid") that have normal values from earlier, appropriate diagnostic tests, should be re-tested at 4 to 6 month intervals avoiding the fall months. The evaluation

should include a physical examination, CBC, serum chemistry, ACTH, insulin concentrations and DST as a baseline profile.[10]

Treatment

Management of PPID is essential to assure appropriate health care of older animals: body clipping, regular hoof and dental care, enhanced nutrition, and intermittent or long term antibiotics may be required. The dopamine agonist, pergolide mesylate (1 to 5 mg/PO/q 24 h/500 kg horse) is most commonly administered since the disease appears to be associated with a loss of hypothalamic dopaminergic innervations. Pergolide should be discontinued in pregnant mares approximately 3 weeks before their anticipated foaling date to avoid agalactia (PRL suppression). The most common adverse effect of pergolide administration is a mild decrease in appetite. Serotonin antagonists (cyproheptadine 0.25 mg/kg/PO/q 12 or 24 h, or 200 to 400 mg/500 kg/PO/q 24 h) are also used alone or in conjunction with pergolide. This therapy is based on the secretagogue effect of serotonin on ACTH in rat pars intermedia.[10,17] The use of cyproheptadine is controversial since results appear to be similar to the clinical improvement obtained with good management alone.

It has been suggested, that a combination of 1 mg of pergolide and 200 mg of cyproheptadine/day accelerates the clinical response to medication, increasing the chances of successfully breeding a mare with history of development of anovulatory follicles or chronic endometritis.[6]

Recent studies have examined the use of trilostane (0.4 to 1.0 mg/kg/q 24 h in feed) in horses with PPID.[10,18] This drug is a competitive inhibitor of 3-ß hydroxysteroid dehydrogenase, an adrenocortical enzyme needed for cortisol production. Even though some treated horses exhibited a decrease in clinical signs, there is reservation toward using this treatment because less than 20% of horses with PPID have adrenocortical hyperplasia.

Aging, obesity, insulin resistance and infertility

Recent studies associate obesity and insulin resistance in horses with development of abnormal reproductive function and laminitis.[19] Equine MS and CD or PPID share traits that provide plausible explanations for the clinically perceived increased incidence of fertility problems in horses.

a) The shared clinical manifestations of both diseases are: aged horse, obesity, regional subcutaneous adiposity (neck, tail head, prepuce), a history, presence or predisposition for laminitis and/or infertility.
b) The shared metabolic alterations include: increased insulin and or insulin resistance, +/_ increased glucose (and in some cases increased leptin and "leptin resistance", cortisol and fatty acid).
c) The shared presence of excessive fat, in the form of obesity or regional adiposity, creates at the cellular-molecular level an increase in "adipocytokines" such as tumor necrosis factor-α (TNF-α). Therefore, PPID and MS may also have an increased level of TNF-α, a pro-inflammatory cytokine which inhibits the proper function of the insulin receptor. Increased fatty acids impair glucose transport through the membrane. Increased 11ß-hydroxysteroid dehydrogenase-1 (11ß-HSD1) converts cortisone to cortisol generating more adipogenesis and IR.[2]

Similar to humans, old horses have been identified as developing a condition referred to as "inflamm-aging". This condition is a persistent low grade, systemic, chronic, inflammation caused by an increased production of serum levels of IL-6 and TNF-α.[20] Increased body fat, associated with obesity, may be a contributing factor since adipose tissue leads to increased production of inflammatory cytokines.[20,21] Adams et al, showed that fat, old, horses between 20 and 28 years old, had greater mRNA expression of TNF-α and INF-γ from lymphocytes and monocytes and had higher serum concentrations of TNF-α protein than thin old horses.[21] The impact of this increase in pro-inflammatory cytokines and infertility is unknown. In humans, elevated TNF-α plays a direct role in the development of obesity-associated insulin resistance and it appears that inflammation may be a key link between obesity and IR in horses as well.[19]

In women, chronic insulin resistance and hyperinsulinemia are associated with increased duration of the follicular phase of the menstrual cycle. Polycystic ovarian syndrome (POS) in women is often associated with obesity and characterized by several clinical signs, neuroendocrine and metabolic alterations. Clinical signs that are diagnostic in women are: ovulatory dysfunction, clinical hyperandrogenism (hirsutism, alopecia, acne) and IR/hyperinsulinemia.[13]

Neuroendocrine disturbances associated with POS include an increase in GnRH pulses that selectively stimulate LH release but not FSH. LH stimulates the expression of the P450c17 cytochrome in the theca cells, increasing the production of 17α-hydroxyprogesterone and testosterone. This hormonal environment (↑LH/↓FSH) impairs follicular maturation, decreases the number of granulosa cells and aromatization ($\downarrow E_2$), and inhibits normal ovulation.[13] Insulin resistance/hyperinsulinemia may have some role since administration of metformin decreases the plasma levels of insulin, LH and androgens in women.[13,22] The increased level of insulin and insulin like growth

factor-1 (IGF-1) directly target the theca cells acting in synergism with LH, stimulating P450c17 cytochrome production and hyperandrogenism. They also decrease the hepatic secretion of the sexual hormone binding globulins (SHBG) and the IFG binding proteins (IGFBP-1) producing hyperandrogenism by increasing the free testosterone fraction and the IGF-1 bioactivity.[13]

There are limited studies in the mare on the endocrine effects of IR, MS, or PPID on reproductive function. One study showed that a great proportion of obese mares continued to cycle during the winter compared to lean mares.[23,24] It was speculated that metabolic alterations in the form of excessive energy imbalance may lead to continuous cycling during winter.[23,25] Later studies found that obese mares had aberrations in the duration of the estrous cycle: longer inter-estrus intervals (36.72±4.77 vs. 26.00±0.54 days), prolonged luteal phases (30.00±5.55 vs. 17.67±0.18 days) and a higher incidence of anovulatory follicles compared to lean mares.[23,26] Progesterone remained elevated for periods of 37 to 78 days in 83% of the obese mares while the longest time progesterone was elevated in lean mares was 22 days.[24] The persistence of elevated serum levels of progesterone in obese mares was thought to be associated with a persistent corpus luteum or luteinization of an anovulatory follicle.[27] Ultrasonic examination of the reproductive tract of these mares showed large, persistent anovulatory follicles during periods of elevated progesterone suggesting that one or more mechanisms involved in ovulation may be suppressed or inhibited.[28] Obese mares exhibited lower levels of serum concentrations of thyroxine and leptin and higher levels of insulin and insulin insensitivity compared to lean mares.

The mechanism of how hyperinsulinemia and IR may disrupt normal reproductive activity is unknown but the following data suggest a direct effect on the ovary rather than the hypothalamic-pituitary-ovary axis. In domestic species, insulin in combination with IGF, promotes follicular development and modulates ovarian steroids secretion as seen in women with POS.[13] Sessions et al., showed that induction of transient insulin resistance in the mare lengthens the interovulatory interval (mean duration 19.57±2.66 vs. 15.57± 0.97 d), creates higher serum peak concentrations of progesterone (12.87±4.04 vs. 8.78±2.23 ng/mL) but does not affect serum LH concentrations (3.9±0.34 vs. 3.29±0.13 ng/mL) when compared to control mares.[29]]

In women, hyperinsulinemia affects the menstrual cycle by alterations of the GnRH-mediated LH release and has a direct effect on the ovary by increasing steroid secretions.[30,31] This phenomenon is well documented in women with POS where there is an arrest of follicular development. Apparently, small follicles that just attained LH receptors, in the presence of insulin and high LH, produce estradiol levels that are similar to levels produced by mature follicles, thereby inhibiting growth and arresting follicles in the immature stage. A similar phenomenon may be occurring in the mare with insulin resistance; however it appears to be due to a direct effect of insulin on the ovary since the hypothalamic-pituitary axis was not affected during an induced short term hyperinsulinemia. Peak concentrations of progesterone were higher in mares experiencing transient IR compared to control mares suggesting an increased steroid secretion by the ovary. It has been proposed that insulin has a stimulatory effect on progesterone production by luteal tissue and this effect is mediated by changes in the developing follicle. In studying the repeatability of hemorrhagic anovulatory follicles (HAF) in ponies, Ginther also deflected the focus from the hypothalamic-pituitary-ovary axis.[32] He showed that follicular diameter, levels of LH and progesterone were similar in ovulating and HAF mares before and at the day of ovulation. The impact of his findings on obese mares remains unknown.

The longer interovulatory interval in obese mares appears to be due to luteinization without ovulation of the dominant follicle (failure to ovulate) extending the luteal phase. One plausible explanation is what happens in women with POS; insulin directly targets the theca cell stimulating P450c17 cytochrome production and hyperandrogenism. Increased androgen inhibits FSH secretion and this hormonal environment (↑androgens/↓FSH) impairs follicular maturation, decreases the number of granulosa cells and aromatization (↓E_2), and inhibits normal ovulation. Further studies need to be performed to confirm this theory by measuring androgens and FSH in mares with persistent IR.

Another explanation, which does not exclude the prior, is that elevated insulin affects follicle remodeling mechanisms necessary for ovulation. Normal follicular growth, ovulation and atresia require remodeling events which are carried out by matrix metalloproteinases (MMP) and tissue inhibition of metalloproteinases (TIMP). Recent studies indicate that exposure to elevated insulin affects the *in vitro* expression of MMP and TIMP in preovulatory and atretic follicles. Moreover, it specifically disrupts the expression of TIMP-2 modifying the ratio of MMP/TIMP expression by equine granulosa cells of large follicles. This alteration may be responsible for the inability of the follicular wall to remodel for development, migration and ovulation.[26,33]

Conclusion

Although the pathophysiology of metabolic diseases such as IR, MS or CD is not completely known, there is increasing evidence that these conditions may adversely impact fertility of the mare. Diagnostic testing for these conditions should be considered in old, obese mares that repeatedly form anovulatory follicles, experience bouts of laminitis or endometritis. While accuracy of diagnostic tests for detecting disease may not be as high as one would

like, a combination of tests along with clinical assessment will identify the vast majority of mares. A strict exercise regimen plus removal of feed stuffs rich in carbohydrates from the diet and supplementation with thyroid hormone and/or pergolide should improve fertility in affected mares.

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The heat is on: what's new for suppression of estrus in mares

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Abstract

Therapeutic suppression of estrus in mares is routinely performed when exhibition of estrous behavior is deemed undesirable or it interferes with intended uses such as recreational and/or performance activities. Various modalities are available for suppression of estrus in mares, each with its own advantages, disadvantages and efficacy (or lack of). This paper will review currently available methods of suppressing estrous behavior in mares, which includes administration of exogenous progesterone/progestins, extending the duration of corpora luteal function, suppressing ovarian follicular activity and ovariectomy. Particular emphasis will be placed on recent studies supporting or refuting the effectiveness of these methods of blocking estrus, so the clinician can identify the most suitable and efficacious method of suppressing estrus for horse owners and their animals.

Keywords: Equine, mare, estrus, estrous behavior, therapeutics

Introduction

A relatively common complaint of horse owners and trainers is variable performance in mares related to the estrous cycle.[1] Similarly, approximately 90% of over 750 veterinarians responding to a survey had the clinical impression the estrous cycle impacted the performance of mares, and the most frequently reported clinical sign associated with an effect of the reproductive cycle on performance was attitude change, while other signs included tail swishing, difficulty to train, squealing, "horsing", excess urination, kicking, and a decrease in performance.[2] In some cases undesirable behavior has been associated with estrus and in other cases with diestrus.[3] It is important to note that some problematic behaviors displayed by mares that are thought to be associated with estrus, are in fact not estrous behaviors; in particular, submissive behavior may be most easily confused with estrous behavior. Submissive behavior includes leaning away from perceived threats, swishing/ringing the tail and actively squirting urine, which collectively can give the impression of estrus.[4] In contrast to submissive behavior, true estrous behavior includes leaning towards the stallion (or other stimulus), a relaxed lifting motion of the tail, stationary/squatting stance and passive urination (full stream or small amounts in spurts).[4] In some mares the signs of true estrous behavior are so strong as to directly impair performance; for example, even under saddle some mares may "break down" and show estrus in response to being around other horses and/or other stimuli.[4] In other mares, the condition may be much more subtle, simply causing owners and trainers to report the mare is less cooperative or attentive during estrus.[1]

In an effort to evaluate the potential for an effect of stage of estrous cycle on the behavior of mares, Hedberg et al.[5] conducted two behavior tests (novel object and isolation) on 12 mares once when they were in estrus and once during diestrus in a cross-over design. Five of the mares served as controls, while the other seven mares were classified as "problem" mares based on their owner's perception of estrus-related behavioral problems. There were no significant differences in the behavioral responses between estrus and diestrus within the control and "problem" groups, nor were there differences in the behavioral responses between the two groups of horses; however, as the authors note, the sample size was small and a cross-over study design may not have been the most appropriate experimental design. Therefore, further work in this area is warranted.

Ideally, a complaint of an estrous cycle-related behavior/performance problem in a mare should be systematically evaluated in order to determine if the problematic behavior is or is not related to a specific phase of the cycle (i.e., estrus or diestrus). A team approach to evaluating and addressing the problem involving behavioral and reproductive expertise may be beneficial.[6] In situations where there is evidence that the problematic behavior is associated with estrus, suppression of estrus may be warranted. It is also common to suppress estrus in situations where the signs of estrous behavior are simply perceived to be associated with performance problems or to preemptively block the behavior to preclude the possibility of an adverse effect of estrus on performance. This paper will review currently available methods of suppressing estrous behavior in mares, with particular emphasis on recent data that support or refute various methods of blocking estrus. The following methods of suppressing estrous behavior will be discussed: 1) administration of exogenous progesterone/progestins, 2) extending the duration of corpora luteal (CL) function, 3) suppressing ovarian follicular activity and 4) ovariectomy.

Administration of exogenous progesterone/progestins

Progesterone

It was first demonstrated in the 1960's that daily intramuscular administration of 100 mg progesterone in oil (0.2 mg/kg) effectively suppressed signs of estrus in mares.[7] Intramuscular administration of 100 mg progesterone in oil to ovariectomized mares produced peak systemic blood levels of approximately 2 ng/mL, which then declined to 1 ng/mL or less 24 hours later.[8] Progesterone in oil is available from several compounding pharmacies; however, the need for daily administration and the potential for soreness at the site of injection are limitations to its use. It has also been demonstrated that intramuscular administration of a compounded long-acting formulation of progesterone containing a total dose of 1.5 g progesterone will maintain blood levels of progesterone above 1.0 ng/mL for approximately 10 days,[9] which is a sufficient level of progesterone to block estrous behavior;[7,8] however, the potential for soreness at the injection site is a limitation to its use, particularly in performance horses.

Altrenogest

Altrenogest (Regu-Mate®, Intervet/Schering-Plough Animal Health, Millsboro, DE, USA) is a synthetic progestin approved for use in horses for suppressing estrus, and is widely considered to be the "gold-standard" method of inhibiting estrous behavior. Daily oral administration of altrenogest at a dose of 0.044 mg/kg (1 mL per 110 lbs of body weight) is very efficacious for suppressing estrus in mares;[10,11] however, the need for daily administration is a drawback to its use. It was recently reported that intramuscular administration of a compounded preparation containing 225 mg or 450 mg of altrenogest in a sustained-release vehicle blocked estrous behavior for approximately 12 and 15 days, respectively, while administration of 500 mg altrenogest in lactide-glycolide microparticles suppressed estrous behavior for approximately 30 days.[12]

Medroxyprogesterone acetate

Although there are anecdotal reports on the use of the synthetic progestin medroxyprogesterone acetate (MPA) for estrus suppression in mares, it was recently reported that intramuscular administration of an initial dose of 1,600 mg MPA followed by 400 mg once weekly for five weeks did not suppress estrous behavior in mares.[13] Similarly, administration of 1,000 mg MPA in an aqueous suspension did not prolong the return to estrus compared to control mares.[12] On a related note, MPA was unable to maintain pregnancy in mares following induced luteolysis when 1,000 mg was administered intramuscularly every seven days.[14] Therefore, the use of MPA for suppression of estrus cannot be advocated.

Hydroxyprogesterone caproate

Although it has not been tested for its efficacy for suppressing estrous behavior in mares, McKinnon et al. demonstrated that intramuscular administration of 500 mg hydroxyprogesterone caproate every other day was unable to maintain pregnancy in ovariectomized mares.[15] Similarly, the same synthetic progestin, repackaged as hydroxyprogesterone hexanoate, though labeled for pregnancy maintenance was unable to maintain pregnancy in mares when 500 mg was administered intramuscularly every four days following induced luteolysis.[14] Therefore, the use of hydroxyprogesterone caproate for suppression of estrus cannot be advocated.

Melengestrol acetate

Melengestrol acetate (MGA), a synthetic progestin, is labeled as a feed additive for estrus suppression in cattle. However, when fed to mares at 10 or 20 mg/day for up to 15 days, it failed to block estrus.[7] It has been suggested higher doses (>100 mg/day) may be effective for suppression of estrus in mares,[16] which may be plausible, since it was recently reported that oral administration of 100 mg or 150 mg MGA to mares during the spring transitional phase significantly hastened the onset of ovulatory activity compared to control mares indicating apparent biological activity of higher doses of MGA.[17] Therefore, further work to evaluate the effect of higher doses of MGA on estrous behavior is warranted.

Implants

A variety of implants containing various progestins, labeled and marketed for use in other species, have been used in mares with the intent of suppressing estrus. The most widely used implant has been Synovex S® (Fort Dodge Animal Health, Ft. Dodge, IA, USA), which contains 200 mg progesterone and 20 mg estradiol benzoate in each dose of eight pellets. McCue et al. were unable to suppress estrus or cyclicity in mares that received 80 Synovex S® pellets.[18] Similarly, although not critically tested for its ability to suppress estrous behavior, Scheffrahn et al. reported that following subcutaneous placement of a implant containing 6.0 mg of the synthetic progestin norgestomet in six mares, two of the mares displayed estrous behavior two days before the implants were removed 10 days after placement.[19] On a related note, placement of five subcutaneous implants containing a total of 15 mg norgestomet was unable to maintain pregnancy in mares following induced luteolysis.[14] Therefore, at this

time, there is no evidence that implants containing progesterone/progestins have efficacy for suppression of estrus in mares, so their use cannot be advocated.

Extending CL function

Intra-uterine glass marble

One alternative to the use of exogenous progesterone/progestins for estrus suppression is intrauterine insertion of a glass ball to extend CL function, which allows continued secretion of endogenous progesterone to block estrus. Nie et al. reported that placement of a 25 or 35 mm sterile glass ball into the uterine body immediately following ovulation resulted in prolonged CL function in seven of 18 (39%) mares that retained the glass ball after insertion (six of 12 mares expelled the glass marble).[20] In mares that developed prolonged CL function following placement of the glass ball, CL function was maintained for approximately 90 days, during which time progesterone levels remained above 1.0 ng/mL and estrous behavior was not exhibited. In non-treated control mares, spontaneous prolongation of CL function occurred in four of 32 (13%) mares. Although placement of a glass ball appeared to be an efficacious means of blocking estrous behavior for an extended period of time, it should be noted that in addition to the 11 mares that retained the glass ball and never developed extended CL function (i.e., continued to cycle normally), three of the seven glass ball treated mares with extended CL function had one or two estrous cycles of normal duration after placement of the glass ball before CL function was prolonged. Therefore, on a "per-cycle" basis the incidence of prolonged CL function was only 11% (7/62 cycles) in the glass ball treated mares compared to 8% (4/50 cycles) in the non-treated control mares, which was not significantly different between groups. Because of its variable efficacy among mares, and the need to physically remove the glass ball when the resumption of cyclical reproductive activity is desired, placement of an intrauterine glass ball does not appear to be an optimal method of suppressing estrous behavior in mares.

In a more recent study, Rivera del Alamo et al. examined the effect of intrauterine placement of a 20 mm water-filled polyproplylene ball on the duration of CL function in mares with the specific objective of investigating two potential mechanisms by which CL function is extended: 1) the intrauterine device induces mild endometrial inflammation that completely blocks or markedly attenuates prostaglandin (PG) F2α secretion (i.e., prevents high magnitude luteolytic pulses) or 2) the physical presence of the device (movement and/or contact with the endometrium) directly mimics the inhibitory effect of a conceptus on PGF2α secretion.[21] Corpora luteal function was extended in nine of 12 mares (75%) with an average duration of 57 days compared to zero of 12 control mares in which the average duration of CL function was 16 days. In six of the nine mares with extended CL function, small accumulations of intrauterine fluid (≤10 mm x 20 mm) were identified during the luteal phase, but no neutrophils or bacteria were recovered on uterine swabs when they were examined during the subsequent estrus. In addition, changes in uterine biopsy scores for inflammation and glandular dilation pre- and post-treatment were similar for control and uterine device mares (with or without extended CL function); therefore, there was no evidence the intrauterine device induced an inflammatory response in the uterus. Based on intensive blood sampling and measurement of PGF2α metabolite (PGFM) levels in the systemic circulation on days 11 to 16 post-ovulation in four control and eight uterine device mares, PGF2α secretion was attenuated in mares with prolonged CL function, with the exception of two mares; one mare showed a single PGFM peak and another showed two isolated PGFM peaks. Because there was no evidence of inflammatory changes caused by the intrauterine device, the authors concluded the physical presence of the device in the uterine lumen somehow mimicked the effect of a conceptus by impairing endometrial secretion of PGF2α; however, the exact mechanism remains unknown.

Administration of exogenous oxytocin

In contrast to using an intrauterine device to extend CL function, administration of exogenous oxytocin during diestrus is an alternative method of blocking luteolysis to prolong CL function. Endogenous oxytocin secretion is involved in regulating prostaglandin PGF2α secretion from the endometrium during spontaneous luteolysis in the mare,[22,23] and although administration of exogenous oxytocin to mares around the time of luteolysis (i.e., days 11 to 15 post-ovulation) stimulates an acute onset of PGF2α secretion, [24-26] when oxytocin is administered in the mid-luteal phase prior to the expected time of luteolysis (i.e., before day 10 post-ovulation) it does not induce PGF2α secretion and often disrupts luteolysis causing prolonged CL function.[25]

Experimentally, continuous infusion of oxytocin using a subcutaneous osmotic minipump from day eight to 20 post-ovulation blocked luteolysis in four of five mares, whereas luteolysis occurred at the expected time in all four control mares that received saline infusion.[27] Although it successfully induced prolonged CL function, continuous infusion of oxytocin to disrupt luteolysis would not be a practical method of long-term suppression of estrous behavior. More recently, Vanderwall et al. showed that twice daily intramuscular administration of 60 units

of oxytocin on days seven to 14 post-ovulation was an efficacious method of disrupting luteolysis, since it caused prolonged CL function through day 30 post-ovulation in six treated mares (Figure 1), whereas six saline-treated control mares underwent luteolysis by day 16 post-ovulation (Figure 2).[28] Progesterone levels fell below 1.0 ng/mL between days 30 and 40 in two of the mares with prolonged CL function, while the other four mares maintained progesterone levels above 3.0 ng/mL through day 40 when blood sampling was discontinued. The cessation of CL function before day 40 in two mares may have reflected a seasonal effect on CL function, since the study was completed at the end of the physiological breeding season when gonadotropin secretion wanes, and CL function (i.e., progesterone secretion) is dependent upon adequate support from endogenous gonadotropin secretion.[29-31] A follow-up study (Vanderwall et al., unpublished) was then performed to compare use of the same dose of oxytocin (60 units) given twice daily compared to once daily on days seven to 14 post-ovulation; CL function was maintained for 50 days post-ovulation in five of seven mares treated twice daily, five of eight mares treated once daily and in one of seven untreated control mares. There was no difference ($P>0.05$) in the proportion of mares with extended CL function between once daily and twice daily administration of oxytocin, whereas collectively oxytocin treatment increased ($P<0.05$) the proportion of mares with extended CL function. An advantage of using exogenous oxytocin treatment to prolong CL function is that it can be readily reversed by the administration of a luteolytic dose of PGF2α, in contrast to the need to physically remove an intrauterine device as described above.

Inducing late-diestrus ovulation

In 2006, Hedberg et al. described the results of a preliminary study in which their objective was to prolong the luteal phase in mares by using human chorionic gonadotropin (hCG) to induce a late-diestrus ovulation to produce a new CL that would be too immature to respond to the luteolytic effects of endogenous PGF2α secretion at the end of diestrus (i.e., day 14 to 15 after the initial ovulation).[32] Mares were randomly assigned to control (n=4) and experimental groups (n=5), and beginning on approximately day eight after ovulation (or last signs of estrous in three mares) their ovaries were examined with transrectal ultrasonography every other day to determine the size(s) of their diestrus follicles. When a diestrus follicle ≥30 mm was detected, control mares were treated with saline and the experimental mares were treated with 3,000 IU hCG IM. After treatment the mares were followed with transrectal ultrasonography for up to 72 hours or until ovulation was detected, and then once weekly for three weeks. Beginning on the day of treatment, blood samples were collected twice weekly for at least one month and then once weekly for another two to four months for progesterone determination. If a mare did not develop a diestrus follicle ≥30 mm during the first diestrus period, they were monitored for a second, and if necessary a third diestrus period.

Three of the nine mares developed a follicle ≥30 mm during the first diestrus period, four mares during the second diestrus period and one mare in the third diestrus period. One experimental mare never developed a diestrus follicle that was ≥30 mm during the three diestrus periods that were monitored, and therefore, could not be treated with hCG. Overall, three out of the four (75%) experimental mares treated with hCG ovulated within 72 hours after treatment with hCG, which resulted in luteal phases that lasted for 58 to 82 days after treatment. None of the control mares ovulated during the luteal phase; however, one control mare had a spontaneously prolonged luteal phase during both a non-treated cycle in which she never developed a diestrus follicle ≥30 mm (CL function was terminated with exogenous PGF2α) and during the subsequent cycle in which she was treated with saline when she had a large diestrus follicle (that did not ovulate).

Although, based on this study, the use of hCG to induce a late-diestrus ovulation looks promising for prolonging CL function, it is important to note that as described above, for some mares (five out of nine) it required multiple estrous cycles to develop a diestrus follicle ≥30 mm. In addition, one mare never developed a large diestrus follicle during the three cycles that were monitored, which precluded her from receiving the hCG treatment. Therefore, in addition to the effort (and expense) of monitoring mares in order to evaluate their suitability for treatment, the fact that some mares may not develop a large enough diestrus follicle to warrant treatment, the use of hCG to induce a late-diestrus ovulation does not appear to be a reliable, "on-demand" method of blocking estrous behavior in mares. It is interesting to note that although the use of hCG was apparently efficacious for inducing ovulation of diestrus follicles ≥30 mm in diameter in this study by Hedberg et al.,[32] previous work by Glazar et al.[33] demonstrated that administration of the GnRH agonist deslorelin acetate failed to induce ovulation and/or luteinization of diestrus follicles > 30 mm in diameter.

Pregnancy

Pregnancy is another means of suspending cyclicity by taking advantage of the natural ability of the conceptus to block luteolysis and maintain CL function/progesterone secretion. Although efficacious, this method has obvious disadvantages that may make it undesirable for many horse owners. In addition to the time and expense

necessary to establish pregnancy, is the need to eventually terminate pregnancy (unless an offspring is ultimately desired). Lefranc and Allen reported that manual transrectal rupture of the conceptus between days 16 and 22 of gestation in 11 mares resulted in continued CL function for at least 60 days in all of the mares, during which time they did not display estrous behavior.[34] Although efficacious, as noted above, terminating a normal, healthy pregnancy may be untenable to many horse owners.

Suppressing ovarian follicular activity

When considering the use of suppression of ovarian follicular activity as a method of blocking estrous behavior in mares, it is important to recognize that mares are unique among domestic animals, because in addition to the ovarian-derived estrogen-induced signs of estrous behavior that occur when progesterone is at a basal level, many seasonally anovulatory (and ovariectomized) mares exhibit paradoxical estrous behavior associated with hormone secretion from the adrenal cortex.[35,36] The intensity of this type of "unseasonable" estrous behavior was judged to be equivalent to the behavior intact cycling mares display during the initial and terminal days of estrus, but less intense than the behavior displayed near ovulation.[35] Such behavioral receptivity to a stallion outside the breeding/ovulatory season that is independent of ovarian estrogen secretion may have developed as a means of maintaining social bonds between a harem stallion and his mares.[35,37] This phenomenon has important implications for the clinical management of estrous behavior in mares, since simply suppressing ovarian follicular activity (or removing the ovaries) and its attendant estrogen production may not ensure the elimination of estrous behavior.

Down-regulation of the hypothalamic-pituitary-ovarian axis with gonadotropin releasing hormone (GnRH) analogs

Although a subcutaneous implant containing the potent GnRH analog deslorelin acetate (Ovuplant®, Fort Dodge Animal Health) was initially developed and found to be efficacious for inducing timed ovulation for the breeding management of mares,[38,39] it soon became evident the implant caused prolonged anovulatory intervals in some mares.[40-42] Subsequent work demonstrated the deslorelin implant caused reduced circulating FSH concentrations and absence of the mid-cycle FSH peak that was associated with a prolonged inter-ovulatory interval.[43] Although problematic for the breeding management of mares, the down-regulating effect of the deslorelin implant on pituitary function has been used clinically to suppress ovarian activity; for example, placement of two deslorelin implants suppressed follicular development in mares used as recipients for oocyte transfer.[44] Fitzgerald et al. reported suppressing ovulation for 30-90 days in 15 of 20 mares that were treated with a subcutaneous implant containing another GnRH analog (goserelin acetate).[45] Collectively, these data indicate that treating mares with a potent GnRH analog may be efficacious for suspending cyclicity and suppressing estrus in mares for extended periods of time. However, as noted previously, since anovulatory mares can show estrous behavior, suppressing follicular activity may not ensure a complete absence of estrous behavior.

Immunologic

Immunizing a mare against GnRH would eliminate the stimulus for gonadotropin release from the pituitary resulting in suspension of cyclicity. Tshewang et al. reported successfully suspending ovarian activity and suppressing estrus for 25-30 weeks in mares after treating with a GnRH vaccine.[46] All of the vaccinated mares recovered from the effect of immunization with normal cyclicity and estrous behavior. Over the next two seasons, each mare also conceived and produced a normal foal. Subsequent studies have confirmed the efficacy of vaccination against GnRH for suppressing ovarian follicular activity; however, not surprisingly, some of the vaccinated mares continued to show estrous behavior in spite of their anovulatory state. It is important to note, that in one study[47] one vaccinated mare had not resumed normal ovarian activity two years after initial vaccination; therefore, although most mares appear to regain ovarian activity within a reasonable period of time post vaccination, some mares may not, which could be particularly problematic. Although a GnRH vaccine is not currently available in North America, commercial preparations are available in Europe and Australia (Equity®; Pfizer Australia Pty Ltd, West Ryde, NSW, Australia).

Ovariectomy

Bilateral ovariectomy may be warranted in some circumstances for permanently eliminating ovarian activity in mares. Although the ovaries can be removed through a ventral abdominal or flank laparotomy, laparoscopy or a colpotomy, at this time the latter two methods are most commonly utilized.[48,49] In an initial report, Hooper et al. described the outcome of the use of bilateral ovariectomy for treatment of objectionable behavior during estrus in 17 mares; they found that after surgery owners reported that behavior was no longer a problem in 14 mares (82%), while the remaining three mares continued to show estrous behavior.[48] Of the 23 mares in that study that underwent bilateral ovariectomy for any reason, eight (35%) continued to show estrous behavior after surgery.

In a recent report, Kamm and Hendrickson evaluated clients' perspectives on the outcome following the use of laparoscopic ovariectomy for behavioral and medical problems in mares.[49] Overall, client satisfaction (rated as very satisfied or satisfied) with bilateral ovariectomy as a treatment for behavioral problems was 78% (18 of 23 of mares). Assessment of outcomes for specific behavioral problems showed that aggression problems improved in 86% of cases; general disagreeable demeanor improved in 81%; excitability improved in 75%; kicking and biting at other horses improved in 73%; problems in training improved in 72%; frequent urination improved in 64%; and problems with other horses improved in 64% of cases. The most common source of dissatisfaction for owners of patients with behavioral problems was lack of behavioral change after surgery, including continued signs of estrous behavior. Although ovariectomy offers the advantage of being a potentially permanent solution to a cycle-related behavior/performance problem, there are significant disadvantages as well. The procedure is relatively expensive, though when compared to repeated hormonal treatments, the cost may, in fact, be comparable. There are also risks associated with the surgical procedure, though newer laparoscopic procedures have significantly reduced them.[48,49] The permanency of the procedure can also be a significant disadvantage because all possibility for future reproduction is eliminated. And as noted above, because of the potential for paradoxical estrous behavior, removing the ovaries may not ensure cessation of estrous behavior. Therefore, this option should be weighed carefully before proceeding. One way of evaluating the potential effectiveness of ovariectomy for a behavioral problem is to evaluate the mare's behavior during either a natural (i.e., seasonal) or induced anovulatory state (e.g., down-regulation with a GnRH agonist); if the problem is not resolved or at least significantly improved during the anovulatory state, it is unlikely ovariectomy will be any more efficacious.

Summary

The primary indication for suppressing estrous behavior in mares is cycle-related behavior/performance problems during estrus. When evaluating an owner/trainer complaint of an estrous cycle-related problem in a mare, it should first be determined if the problematic behavior is or is not related to a specific phase of the estrous cycle. In order to thoroughly evaluate the mare, additional expertise may be needed in the form of consultation with or referrals to behavior and/or reproduction experts. Once a behavior/performance problem is confidently defined as being related to estrus, the previously discussed methods (with proven efficacy) of suppressing estrous behavior can be considered for use. Each method has advantages and potential disadvantages, that should be weighed for each individual animal/owner/trainer.

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Figure 1. Serum progesterone concentrations from the day of ovulation (day 0) through day 40 after ovulation in six mares treated with 60 units oxytocin intramuscularly twice daily on days seven to 14 after ovulation (reprinted with permission from JAVMA 2007;231:1864-1867).

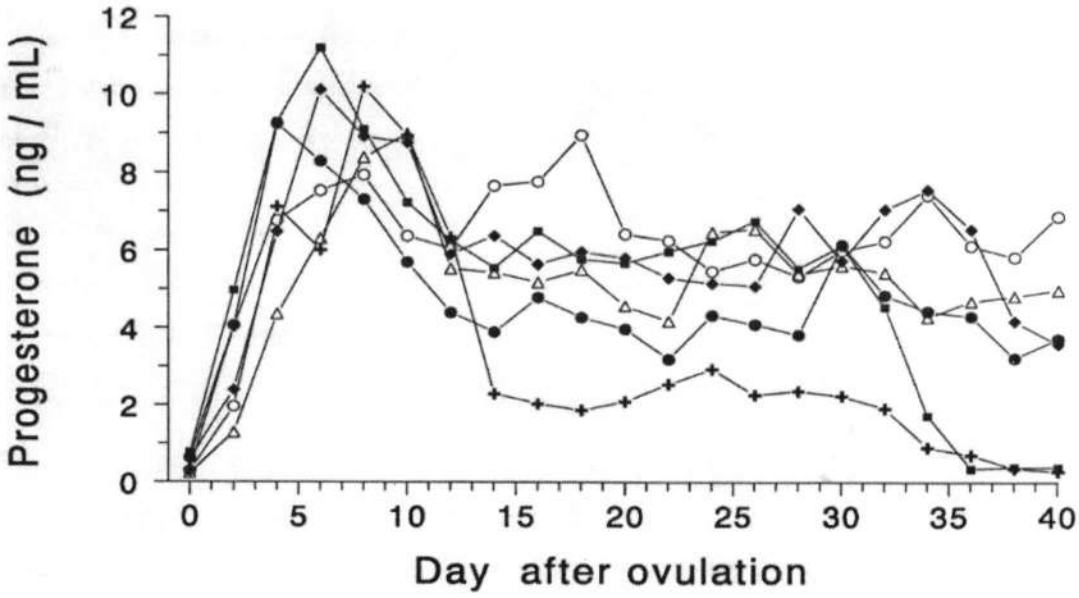
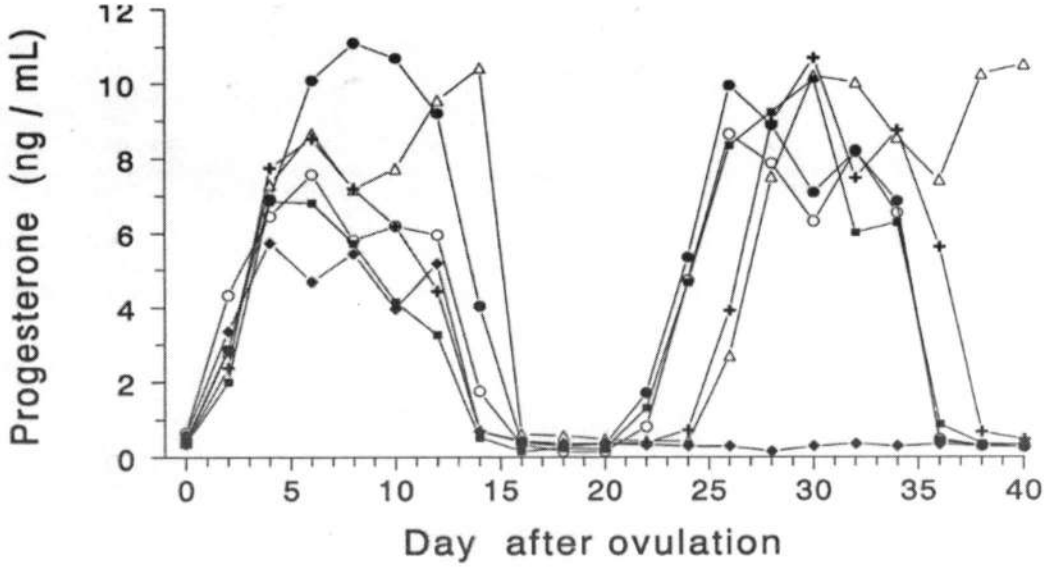


Figure 2. Serum progesterone concentrations from the day of ovulation (day 0) through day 40 after ovulation in six mares treated with 3 mL sterile saline intramuscularly twice daily on days seven to 14 after ovulation (reprinted with permission from JAVMA 2007;231:1864-1867).



Influence of delivery method on neonatal canine viability parameters

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While many important clinical parameters are used to asses viability, clinical outcome and pathology have been well-established in human neonates, foals, calves, and piglets; they are virtually unknown during the immediate postpartum period in the puppy. The aim of this study was to develop a database on neonatal puppies delivered via vaginal delivery, elective cesarean section, and emergency cesarean section and correlate the values with subjective assessment of viability. Preliminary studies involved 9 puppies from three litters that were born by natural vaginal delivery (Group A) and 3 puppies from one litter that were delivered by emergency cesarean section (Group B). Hematology samples were collected via venipuncture of the jugular vein. All samples were collected within 26 minutes of birth. Significant differences were noted between Group A and B in serum glucose concentrations (111±38 vs 54±17 mg/dl), blood urea nitrogen (22.8±5.1 vs 8.0±7.1 mg/dl), and pO2 (34.1±12.0 vs 53.7±18.2 mmHg). However, all values were within normal adult ranges. There were no significant differences between the rest of the measured values but the following trends were observed: APGAR scores and Doppler blood pressures were higher in Group A than B, while pCO2 and sO2 were higher in Group B than A. The ranges in serum glucose, sodium, chloride, lactate, HCO3, base excess, and sO2 were much wider in Group A than B while blood pressure ranges were wider in Group B. Lactate concentrations were higher, while pH and HCO3 levels were lower, in all puppies when compared to normal adults, indicating lactic acidosis and tissue hypoxia. There was no difference in the degree of lactic acidosis between Groups A and B and base excess was lower in all puppies. Interestingly, there was no correlation between lower APGAR scores and the degree of acidosis, blood glucose levels or any other parameter measured. In conclusion, significant differences in serum glucose, blood urea nitrogen and pO2 exist between puppies delivered via natural vaginal delivery and via emergency cesarean section. Furthermore, these results suggest that significant lactic acidosis is present in puppies immediately after birth, but that there is no significant difference in degree of acidosis between puppies delivered via natural vaginal delivery and emergency cesarean section. However, the presence of acidosis in all puppies did not appear to impact viability as all puppies thrived past the neonatal period. Further studies will aim to expand the number of puppies assessed in Groups A and B, as well as include puppies from another group, planned cesarean section, and examine how long after birth lactic acidosis is present.

Keywords: Pediatrics, neonatal, dog, hypoxia, cesarean section

Testosterone decreases to basal values within 24 hours following castration in alpacas

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The objective of this study was to compare serum testosterone concentrations ([T]) in adult male alpacas (*Vicugna pacos*) following administration of human chorionic gonadotropin (hCG) before and after castration. This information is of diagnostic significance for the determination of retained testicular tissue following castration or in the case of cryptorchidism. Jugular blood samples were collected from nine adult alpacas prior to intravenous administration of hCG (2500 IU; Chorulon®, Intervet, Millsboro, DE, USA) as well as 2, 4 and 6 post-hCG. Alpacas were castrated using routine surgical techniques 24 h post-hCG. Testes were removed from the vaginal tunica, the epididimydes were removed, and the testes were weighed. A post-castration hCG response test was performed 24 h after castration (48 h from the previous hCG response test). Serum [T] was measured using a double antibody radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA). The assay sensitivity was 0.04 ng/ml and the intra-assay and inter assay CVs were <10%. All of the samples were tested within one assay. The mean [T] at each treatment time were compared using an analysis of variance. In addition, [T] were compared to testicular weight using linear regression. Significance was defined as $p<0.05$. The results for individual animals are summarized in the table below. The [T] was significantly increased from pre-hCG values by 2 h post-hCG. The highest mean [T] occurred 6 h post hCG, however [T] did not differ significantly between 2, 4 and 6 h post hCG. At 24 h post-castration (48 h post-hCG), the [T] was below the limits of detection. There was no significant correlation between [T] at any time point and testicular weight. In summary, serum [T] is not correlated with testicular weight and falls to undetectable values within 24 h following castration in alpacas.

		Serum Testosterone Concentrations (ng/mL)				
ID	**Testicular Weight (g)**	**Pre-hCG**	**2 h Post-hCG**	**4 h Post-hCG**	**6 h Post-hCG**	**48 h Post-hCG (24 h Post-castration)**
1	23.49	3.23	10.32	15.53	20.37	<0.04
2	22.62	7.72	14.30	14.21	18.58	<0.04
3	21.99	3.62	5.98	10.43	8.53	<0.04
4	28.80	4.22	9.89	9.74	13.25	<0.04
5	28.09	4.44	7.15	6.33	6.96	<0.04
6	17.94	7.14	10.65	16.21	19.23	<0.04
7	14.69	0.67	3.81	6.26	4.71	<0.04
8	28.91	9.12	17.66	20.89	24.91	<0.04
9	36.16	5.60	18.48	19.83	24.94	<0.04
$\bar{X}$	**24.74**	**5.08**	**10.92**	**13.27**	**15.72**	
SD	**6.48**	**2.60**	**5.06**	**5.40**	**7.64**	

Keywords: Castration, hCG, alpaca, testes, testosterone

Investigation of ovulation induction in alpacas with acupuncture

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Acupuncture has been used to induce ovulation in several animal species, including induced ovulators such as the rabbit. There is no published information on induction of ovulation in alpacas using acupuncture. A pilot study was conducted to test the hypothesis that acupuncture induces ovulation in alpacas.

Four alpacas 8-14 years old were used in a cross-over design to compare two treatments, GnRH and acupuncture, for ovulation induction during 2 follicular wave cycles. Treatment with GnRH (Cystorelin® 50μg/ml IM, Merial, Duluth, GA, USA) or acupuncture was performed when the alpacas had uterine edema and an ovarian follicle 8-10mm in diameter as detected by ultrasonography (US). Ovulation was confirmed using US to detect the presence of a corpus luteum on the ovary. All alpacas received cloprostenol (Estrumate®, 250 μg, Intervet/Schering-Plough Animal Health, Kenilworth, NJ, USA) intramuscularly 7 days after induction of ovulation and were subjected to the second treatment during the next follicular wave (1 to 2 weeks later).

Acupuncture consisted stimulating the acupoints by aquapuncture; injecting 3 ml vitamin B_{12} (cyanocoblamin, 1000 μg/ml, VEDCO, Inc, St. Joseph, MO, USA) at the bladder (BL) and stomach (ST) acupoints (BL 22, BL 51, BL 23, BL 52, ST 25), and Yan-chi. The acupuncture points used in this study are utilized to treat various causes of infertility.

Blood samples were collected by jugular venipuncture from each animal before treatment and 7 days after treatment. Sera were stored at -20°C until assayed for progesterone concentration (P4). P4 >2ng/ml was considered as evidence of ovulation and development of a corpus luteum.

Ovulation was induced by GnRH in all alpacas, as evidenced by detection of a corpus luteum with US and a concomitant increase in P4. None of the alpacas ovulated following acupuncture treatment. Various reasons are possible for the failure of ovulation induction using acupuncture in this study. Contrary to other reflex ovulators such as the rabbit where acupuncture was found to be useful for induction of ovulation, camelids do not have a neuroreflex mechanism for induction of the LH surge. An ovulation-induction factor present in seminal plasma stimulates an LH surge similar to that induced by GnRH. As expected, GnRH induced ovulation in all cases selected based on follicular size and uterine edema. The lack of response to acupuncture in the present study could be due to other factors. Acupuncture was shown to cause a significant increase in ß-endorphin levels that lasts for up to 24 hours. The role of neuropeptides, including ß-endorphin, in the regulation of GnRH secretion has been reported. Other acupuncture approaches need to be investigated in order to determine the usefulness of this technique in camelids.

Keywords: Acupuncture, ovulation induction, alpaca

Synchronization of follicular waves with progesterone/estrogen combination before superstimulation with pFSH in alpacas

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Ovarian response to superstimulation with pFSH depends on control of follicular waves before initiation of treatment. We hypothesized that a combination of progesterone and estrogen treatment would produce better synchronization of follicular waves in alpacas than progesterone alone. We tested this hypothesis in two experiments. In experiment one; adult females (five per group) were given no treatment (control), daily progesterone injection (25 mg in oil, IM) for 10 days (P4 group) or combination of progesterone (25 mg) and estrogen (5 mg) IM for 10 days (P/E group). Ovarian activity was monitored daily by transrectal ultrasonography (US) (Aloka 500, Wallingford, CT, USA, with 7.5 MHz linear transducer) until emergence of a dominant follicle (6 mm in diameter). Time between the end of treatment and dominant follicle emergence was compared by ANOVA. In experiment two, adult females (10 per group) were given either P/E for 10 days (group A) or GnRH (Cystorelin ®, Merial, Duluth, GA, USA, 50 mcg, IM) followed by P/E for six days and P4 alone for four days (Group B). After treatment, all females received five days of pFSH BID (20/20 mg, 15/15 mg, 10/10 mg, 10/10 mg and 10/10 mg). Follicular growth was monitored every 48 hours and ovulation induced with hCG (Chorulon®, Intervet/Schering-Plough Animal Health, Millsboro, DE, USA, 1000 IU, IV) when follicles were between 6 to 8 mm in diameter. Ovulation was verified by US seven days after hCG treatment.

In experiment one, follicular activity continued during the observation period in the control animals and in three alpacas in the P4 group. None of the alpacas in the P/E group had follicles greater than 2 mm in diameter on the 8th day of treatment. The mean interval in days and standard deviation (SD) to emergence of a dominant follicle was 5 (3.8), 4.2 (1.8) and 7 (0.7) for the control, P4 group and P/E group, respectively. In experiment two, all females responded to pFSH. However, Group A had a poor ovulation rate and a higher incidence of anovulatory follicles compared to Group B. Group B had a uniform follicular population one day after the end of the pFSH treatment. The number of follicles was difficult to ascertain in some females and ranged from five to greater than 12 per ovary. These preliminary results indicate that treatment with a combination of P/E may be an option for control of follicular activity in alpacas before initiation of superstimulation treatment with pFSH. Administration of GnRH before P/E treatment provides better synchronization of follicular waves probably through the elimination of dominant follicles. Studies are underway to determine the reason for ovulation failure in some of the stimulated females. Factors being studied include dosage of progesterone, estrogen, pFSH and follicle size at the time of ovulation induction.

Acknowledgements:
Dr. Picha was partially funded by the SFT/ACT Zemjanis outreach fund

Keywords: Superovulation, synchronization, anovulatory follicles, alpaca

Oxytocin therapy immediately after parturition does not reduce the incidence of retained fetal membranes or improve reproductive performance in crossbred Zebu cows.

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Oxytocin plays a crucial role during parturition and uterine involution. Many pharmaceutical companies recommend the administration of oxytocin to prevent retained fetal membranes (RFM) and improve reproductive performance (RP) in cows. However, there are limited reports to support this approach, and the results are contradictory. The objective of this study was to determine the effect of oxytocin therapy after parturition on the RFM incidence and RP in dual purpose cows under tropical conditions. Five hundred thirty six multiparous, crossbred Zebu cows were randomly assigned to two groups: Oxy (n=280): 30 IU of oxytocin (Pituifral® C.A. Laboratorios Asociados, Venezuela) injected i.m. immediately after normal parturition, and again 6 hours later and C (n=256): control. Expulsion of fetal membranes was evaluated 24 hours after delivery. Cows were subsequently inseminated12 hours after they were detected in estrus. Data were analyzed using proc logistic and GLM (SAS®, Cary, NC,USA). Oxytocin had no effect on the incidence of RFM (4.6 vs 3.1% for Oxy and C, respectively, $P>0.05$). Cows in Oxy and C had similar conception and overall pregnancy rates (54.0 vs 47.8% and 75.4 vs 73.4%, respectively, $P>0.05$). There were no differences between Oxy and C for calving to first estrus (83.6 ± 3.7 vs 77.2 ± 3.8 days) and calving to conception intervals (113.6 ± 5.0 vs 110.5 ± 5.2 days) anestrus (13.6 vs 13.7%), repeat breeding (21.8 vs 20.7%) and culling rates (15.7 vs 16.4%). Oxytocin therapy after parturition did not reduce the incidence of RFM or improve RP in crossbred Zebu cows under tropical conditions.

Keywords: Oxytocin, calving, reproductive performance, Zebu, cows

Estrus-AI interval and fixed-time AI pregnancy rates in beef cows inseminated with fresh extended or frozen thawed semen

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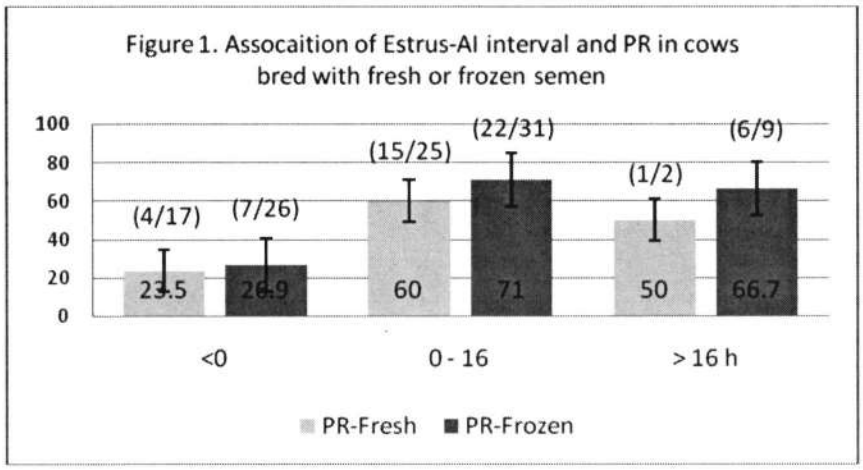
We hypothesize that fresh extended semen will improve the AI pregnancy rate compared to frozen thawed semen due to its increased longevity in female reproductive tract. The objective of this trial was to determine the effect of semen type on fixed-time AI pregnancy rate in relation to estrus-AI interval in beef cows synchronized with progesterone based fixed-time artificial insemination (AI) protocols. Angus cross beef cows (N=180) were synchronized with CO-Synch-CIDR protocols for a fixed-time AI. Briefly, cows were synchronized with 100 μg of gonadotropin releasing hormone (GnRH) + controlled internal drug release insert (CIDR) on Day 0, 25 mg of $PGF2_{\alpha}$ and CIDR removal on Day 7 and 100 μg GnRH on Day 10 (67 h from CIDR removal) at AI (CO-Synch-CIDR). A subset of cows (N=110) received a Heatwatch pressure sensor at CIDR removal to determine the time of estrus. Cows were divided into two groups, inseminated at 47h (early) and 67h (late) from CIDR removal with either 3 million cells of fresh extended or 20 million cells of frozen thawed semen.

Results indicated that cows inseminated at 67h had numerically higher fixed time AI pregnancy compared to cows inseminated at 47h [44.4% (40/90) vs. 33.3% (30/90); P=0.13]. Cows inseminated with frozen thawed semen had similar fixed time AI pregnancy compared to fresh extended semen [40.8% (31/76) vs. 37.5% (39/104); P=0.66]. AI-estrus interval was divided into three groups < 0 h (AI occurred before estrus), 0 to 16 h and > 16 h (AI occurred 16 h after estrus). The AI pregnancy outcome for fresh semen for the 3 estrus-AI intervals was similar to frozen semen (Figure 1).



In conclusion, the fresh semen did not improve the pregnancy rate compared to frozen semen in relation to estrus-AI interval.

Keywords: Beef cows, semen type, fresh semen, synchronization, pregnancy rate

Accuracy of pregnancy specific protein-b test for early pregnancy diagnosis in dairy cattle

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Early pregnancy diagnosis is vital for efficient reproductive management of cattle. Ideally, the diagnostic test used should correctly identify both pregnant and non-pregnant females. The objective of this study was to evaluate the accuracy of the pregnancy specific protein B test (PSP-B) for early pregnancy diagnosis in dairy cattle. Plasma samples from two hundred forty six lactating cows more than 80 days postpartum were collected three times at days 28, 30 and 35 after breeding (estrus= day 0). Each plasma sample was analyzed in duplicate using an ELISA test for PSP-B. Test results for PSP-B were reported as: pregnant, non-pregnant or uncertain (repeat open or repeat pregnant). At the same time, each female was examined by transrectal ultrasonography (TRUS) with a linear 5-7.5 MHz transducer. TRUS was used as a criterion standard test for comparison with PSP-B results. A positive pregnancy diagnosis was made when an embryo plus extra embryonic membranes was observed by TRUS. The agreement between PSP-B and TRUS diagnoses was compared by using Kappa values. The prevalence of pregnancy at days 28 determined by TRUS was 46.3% (114/246). Sensitivity of PSP-B test at days 28, 30 and 35 was 93.9%, 96.0% and 97.2%, respectively ($P>0.05$). Specificity of PSP-B test for the same days was 95.5%, 93.9% and 93.6% ($P>0.05$). The positive predictive values for days 28, 30 and 35 were 94.7%, 92.2% and 92.0%, respectively ($P>0.05$). The negative predictive values for the same days were 94.7 %, 96.8% and 97.8% ($P>0.05$). The accuracy of PSP-B at days 28, 30 and 35 was 96.0 %, 95.4% and 97.5%, respectively ($P>0.05$). However, when compared at the same days with TRUS differences were detected ($P<0.001$). The percentage of uncertain samples among the entire number of specimens analyzed was 5.6% (40/721). A significant reduction in the percentage of samples that required repetition from days 28, 30 to 35 post-AI was detected [8.5 % (21/246), 4.8 % (11/229) and 3.3 % (8/246), respectively ($P<0.01$)]. Kappa value at days 28, 30 and 35 was 0.89 (CI 95%: 0.84-.95), 0.89 (0.84-0.95) and 0.90 (0.85-0.96) ($P>0.05$). It was concluded that the agreement between PSP-B and TRUS was very good. PSP-B test was not a 100% accurate test of pregnancy compared with TRUS at days 28, 30 or 35 after breeding. False negative results were due to low levels of PSP-B in pregnant animals. False positive results were due to persistence of pregnant levels of PSP-B in females with pregnancy loss.

Key words: Cattle, pregnancy diagnosis, PSP-B, transrectal ultrasonography, accuracy

Induction of fertile estrus in bitches using eCG followed by hCG

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The aim of this study was to assess the efficacy of eCG followed by hCG to induce a fertile estrus and a CL capable of maintaining pregnancy in the bitch. Twenty two healthy bitches (Sherman Sheepdog; n=4, Labrador, n=3; Pug, n=9, and West Highland, n=6) aged between 2 and 5 yr, in late anestrous were used in a randomized design. Bitches were divided into two groups. Group I, received a single dose of eCG (50 IU/kg, im; Novormon®, Syntex SA, Argentina) followed seven days later by a single dose of hCG (500 IU, im; Ovusyn®, Syntex SA, Argentina; TRT) and Group II, received 1 ml of saline solution (PLA, im). In TRT group, blood samples were taken to measure serum P_4 concentrations before treatment to confirm anestrous, and after first day of diestrus to confirm ovulation by presence of a CL. In PLA group, anestrus was estimated by date of previous estrus. In both groups, estrus behavior (score 1-3) was recorded and vaginal cytology (score 1-5) was obtained before treatment to confirm anestrus and after treatment to confirm estrus or anestrus (PLA, every other week, up to 4 months). All samples were centrifuged and stored at -20°C until P_4 was measured by solid RIA. Data were analyzed by ANOVA. Data are represented as least square means ± SEM. Significance was defined as $p<0.05$. Before treatment, vaginal cytology scores were similar between TRT and PLA (1.21±0.09 vs. 1.00±0.12, $P<0.18$). After treatment, vaginal cytology scores and estrus behavior scores were higher in TRT compared to PLA (4.78±0.09 vs. 1.00±0.09, $P<0.001$; 2.92±0.05 vs. 1.00±0.07, $P<0.001$). While all bitches in TRT group had estrus behavior, none in PLA group had estrus behavior (14/14 [100%] vs. 0/8 [0%], $P>0.001$). In addition, bitches in TRT group had shorter intervals from treatment to estrus compared to PLA group (4.14±3.38 vs. 74.5±4.48 days, $P<0.001$). Serum P_4 concentrations before TRT were 0.72±0.31 ng/ml and after TRT were 22.85±4.27 ng/ml. The interval from TRT to first day of diestrus was 16.64±0.63 days. Ninety-three percent (13/14) of the bitches were bred (AI, n=6; natural, n=7), 77% (10/13) became pregnant and whelped 3.62±0.41 puppies. From this trial we conclude that 50 IU/kg of eCG combined seven days later with 500 IU of hCG was effective to induce a fertile estrus and corpora lutea capable of maintaining pregnancy in bitches.

Keywords: Bitch, estrus induction, eCG, hCG, pregnancy.

Effect of 2-hydroxypropyl-□-cyclodextrin as a cryoprotectant for porcine sperm on post-thaw rates of acrosomal exocytosis, in vitro fertilization, and embryo development

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The sensitivity of porcine sperm to cryodamage has interfered with the development of a commercially viable method for boar semen cryopreservation. We have previously shown that the addition of the cholesterol shuttle, 2-hydroxypropyl-□-cyclodextrin (HBCD), to an egg yolk-based extender at 40 or 60 mM improves post-thaw porcine sperm viability (Galantino-Homer et al., 2005, Theriogenology 64:805). We propose that HBCD reduces cryodamage by stabilizing sperm membranes. The objective of this study was to test the hypothesis that HBCD addition to porcine semen extender prior to cryopreservation increases the post-thaw percentage of intact acrosomes and has no detrimental effect on in vitro fertilization or embryo development. Porcine sperm were frozen into pellets on solid CO_2 in an extender containing 20% egg yolk (BF5) and 0, 20, 40, or 60 mM HBCD. Pellets were thawed in Beltsville Thawing Solution (BTS) at 37°C for 1 min, diluted into either BTS or calcium-containing capacitation medium (CM+) and either fixed immediately (0 min) or incubated for 5 and 15 min at 39°C before fixing with 4% paraformaldehyde. The percentage of intact acrosomes was evaluated using Coomassie G-250 staining and light microscopy. Three hundred sperm were counted per slide, data given as mean ± SD, n=5, significance set at $p<0.05$ as evaluated by ANOVA followed by Tukey test. The percentage intact acrosomes immediately post-thaw was significantly increased by inclusion of 40 or 60 mM HBCD in BF5 extender, at 26.4 ± 9.9% and 33.8 ± 7.4%, respectively, compared to 10.2 ± 9.1% (0 mM HBCD) and 18.4 ± 13.3% (20 mM HBCD). Addition of 60 mM HBCD significantly increased the percentage of intact acrosomes following transfer to CM+ (18.0 ± 8.5% vs. 0.8 ± 1.3% without HBCD), consistent with reduced calcium-induced acrosomal exocytosis. In vitro fertilization was performed using in vitro matured porcine oocytes and porcine sperm from one boar cryopreserved in BF5 containing 0, 25, or 50 mM HBCD, normalized for viable sperm number. As shown in the table, no significant differences ($p=0.1$) in percentage of blastocysts or blastocyst cell numbers were obtained for this boar.

Table: Effect of HBCD concentration on percentages of oocytes developing to blastocyst stage.

Ejaculate	0 mM HBCD	25 mM HBCD	50 mM HBCD	Total % blast (n)
1	16.7	10.0	8.5	11.7 (179)
2	23.3	18.3	18.3	20.0 (180)
3	5.0	10.2	20.0	12.6 (119)
Total % blast (n)	17.9 (140)	12.8 (179)	15.1 (159)	(478)
Blastocyst cell # (n)	45.8 ± 5.0 (12)	35.2 ± 4.5 (13)	32.4 ± 2.9 (9)	

We conclude that the inclusion of 40-60 mM HBCD in BF5 extender reduces post-thaw cryodamage of porcine sperm and does not significantly alter in vitro fertilization and embryo development.

Acknowledgement
Supported by a grant from the Pennsylvania Dept. of Agriculture (ME 446735).

Keywords: Sperm, acrosome reaction, porcine, cryopreservation, cyclodextrin

Uropathogenic virulence factor FimH facilitates binding of pyometra-causing *E. coli* to canine endometrium

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Pyometra is a prevalent uterine infection that affects intact middle-aged bitches and *Escherichia coli* is the most common isolate. The adhesin FimH is an important virulence factor which contributes to colonization of the urinary tract by uropathogenic *E. coli*.

The objective of this study was to demonstrate that FimH also facilitates binding of *E. coli* to canine endometrium.

Our hypothesis was that disruption of *fimH* expression would lead to a reduction in bacterial binding to uterine epithelial cells. An *E. coli* strain (P4), isolated from a clinical case of canine pyometra, was demonstrated by polymerase chain reaction to carry the gene encoding FimH but no other known *E. coli* adhesins. The chromosomal gene *fimH* was insertionally inactivated with an antibiotic resistance cassette to generate a knock-out mutant (Δ*fimH::Kan*). The P4 wildtype strain (wt) and Δ*fimH::Kan* were further transformed with an expression vector encoding for a green fluorescent protein (GFP; Clontech Laboratories, Palo Alto, CA, USA).

Adhesion assays were used to compare the binding of the wt and Δ*fimH::Kan* to canine endometrium *in vitro*. Anestrus uteri from five bitches were obtained from routine hysterectomies and full-thickness samples were collected using a 6 mm biopsy punch. Tissue samples from each uterus were washed separately in PBS and incubated with P4 wt or Δ*fimH::Kan*, or with PBS as a negative control. After washing, tissue samples were either frozen in liquid nitrogen or homogenized and plated on nutrient agar for determination of colony forming units (CFU)/g of tissue. Thin sections of frozen samples were evaluated for the presence of green fluorescent bacteria.

Adhesion of both bacterial strains to the endometrium was observed by fluorescent microscopy but Δ*fimH::Kan* was considerably less adherent than the wt. This finding was confirmed by viable bacterial cell counts as determined by CFU/g tissue. Binding of Δ*fimH::Kan* was only 3% of that of the wt. The mean difference in binding between the two strains on the log10 scale was 2.5 (SD 0.37) ($p < 0.001$ as per paired t-test). Complementing the mutation in Δ*fimH::Kan* restored the phenotype of the wt binding.

In summary, we demonstrated that disruption of the *fimH* gene in the pathogenic *E. coli* P4 strain significantly reduced bacterial binding to canine endometrium *in vitro*. Future studies targeting uropathogenic virulence factors to prevent binding of *E. coli* to the endometrium might reduce the incidence of pyometra in dogs.

Keywords: Dog, *E. coli*, FimH, uropathogenic virulence factors, pyometra

The effects of dexamethasone and prednisolone on pituitary and ovarian function in the mare

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Persistent mating induced endometritis (PMIE) is among the most common causes of infertility in the mare. Recently, it was reported that glucocorticoids used to modulate the post-mating inflammatory response resulted in an increase in pregnancy rates. The objective of this study was to evaluate the effects of repeated treatment with glucocorticoids on pituitary and ovarian function in mares. Eighteen cycling Quarter Horse mares in early estrus were randomly assigned to one of three treatment groups: dexamethasone 0.05 mg/kg IV BID, prednisolone 0.5 mg/kg PO BID, or placebo for five days. Mares were examined by ultrasound daily to evaluate reproductive function. Blood samples were collected to measure luteinizing hormone (LH), progesterone, and cortisol levels. Dexamethasone treatment resulted in greater ($p < 0.05$) suppression of endogenous cortisol (9.4 ± 1.1 ng/mL) compared to prednisolone (41.9 ± 4.0 ng/mL) or placebo mares (32.4 ± 3.8 ng/mL). Mares treated with dexamethasone exhibited significantly lower uterine edema scores than prednisolone or placebo treated mares after 24 hours. A significant reduction in ovulation rate was noted in dexamethasone treated mares (2/5, 40%) compared to prednisolone (5/6, 83%) or placebo treated (6/6, 100%) mares. An absence of an LH surge was observed in 3 of 5 dexamethasone treated mares and 1 of 6 prednisolone treated mares. In conclusion, repeated administration of dexamethasone was associated with decreased uterine edema, suppression of LH and a high rate of ovulation failure. It is recommended that treatment with dexamethasone be limited to one or two days in the management of PMIE.

Keywords: Equine, ovulation failure, prednisolone, dexamethasone

Age effect on gene expression and mitochondrial DNA in the equine oocyte and follicle

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Reduced fertility in aged mares is linked to declining oocyte quality. Oocyte viability is dependant on the ability of oocytes to remain in meiotic arrest until the initiation of maturation and timing of oocyte and follicular maturation prior to ovulation. We hypothesized that aging is associated with quantitative and temporal differences in expression of genes controlling oocyte meiotic arrest and resumption, paracrine factors secreted by oocytes, and lower mitochondrial numbers, ultimately resulting in a dissociation of oocyte and follicular maturation. Objectives of this study were to clone and determine quantitative and temporal differences in mRNA content of the LH receptor (LHR), amphiregulin (AREG) and epiregulin (EREG) in granulosa cells; phosphodiesterase (PDE) 4D in cumulus cells; and PDE3A, G protein coupled receptor (GPR) 3, growth and differentiation factor (GDF) 9, bone morphogenetic protein (BMP) 15, and mitochondrial DNA (mtDNA) in oocytes during in vivo maturation in young (3-12 yr) and old (>20 yr) mares. Oocytes and follicular cells were collected by transvaginal follicular aspirations. Follicle maturation was induced in estrous mares by injection of 750 μg of recombinant equine LH (reLH) when a follicle > 30 mm was observed. Aspirations were conducted at 0, 6, 9 and 12 h after reLH administration. Total RNA was isolated from single denuded oocytes and associated cumulus and granulosa cells. For each gene, mean mRNA copy number per time point and age group were compared by ANOVA and Fisher's LSD. Regression coefficients were generated to compare oocyte mitochondrial numbers and correlations between gene expressions within age groups. Expression of LHR mRNA in granulosa cells was different ($p<0.05$) between age groups. Young mares displayed a significant drop in LHR mRNA between 0 h and 6, 9 and 12 h, while the pattern of expression in old mares was similar ($p>0.05$) among times and higher ($p<0.05$) at 6 h than in young mares. Expression of AREG mRNA in granulosa cells peaked ($p<0.05$) at 9 h, with the magnitude of expression at 6 and 9 h higher ($p<0.05$) in old than young mares. Similarly, EREG expression peaked ($p<0.05$) at 9 h in young and old mares but was higher ($p<0.05$) for old mares. Expression of PDE4D peaked ($p<0.05$) at 6 and 12 h in old and young mares, respectively. The patterns of expression of GPR3 for oocytes of young and old mares were different and peaked ($p<0.05$) at 9 and 12 h, respectively. Magnitude of expression of PDE3A for oocytes of old mares at 6 and 9 h was higher ($p<0.05$) than in young mares. Expression of GDF9 and BMP15 was different ($p<0.05$) between ages. Mean expression of both genes in the old group was similar over time; however in young mare oocytes, maximum ($p<0.05$) expression was at 6 h. Correlation coefficients between GDF9 and BMP15 for old and young mares were 0.94 and 0.99, respectively. Numbers of copies of oocyte mtDNA did not vary in young mares; however, there was a temporal decrease ($p<0.05$) of oocyte mitochondrial copy numbers in old mares. The main effect for age for mtDNA was similar for old and young mares. Our results support an asynchrony of follicular and oocyte maturation in old mares, which could affect oocyte viability and fertility.

Keywords: Equine, mRNA, aging, oocyte

Expression of anti-Müllerian hormone in equine endometrium

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Classically, anti-Müllerian hormone (AMH) has been characterized for its role in the regression of the Müllerian ducts during male sexual differentiation. However, it is clear that AMH plays an important role after birth in regulation of normal function of the testis and ovary, and a recent publication described the expression of AMH and its receptor in human endometrium. The objective of the present study was to examine the expression of AMH in equine endometrium based upon immunohistochemistry and reverse transcription-PCR (RT-PCR). Mares (n = 6) were examined daily by palpation per rectum and transrectal ultrasound to determine stage of the estrous cycle. Endometrial samples obtained by biopsy at estrus and at Day 10 postovulation were fixed in formalin and snap frozen for subsequent mRNA isolation. Fixed tissues were embedded in paraffin and sectioned prior to analysis by immunohistochemistry using a goat polyclonal primary antibody directed against a C- terminal peptide antigen from human AMH (Santa Cruz Biotechnology; Santa Cruz, CA; USA) followed by a biotinylated second antibody (donkey anti-goat IgG) and detection using the Vectastain ABC detection kit (Vectorlabs; Burlingame, CA, USA). Specificity of the immunolabel for AMH was demonstrated by use of the corresponding blocking peptide. To confirm expression of AMH in the equine endometrium, RT-PCR was performed using equine specific oligonucleotide primers for AMH (forward: 5'-GAGCTGCAGGCGGCGGCG-3'; reverse: 5'-GGCCCCCGCGTTGCGCTG-3'). Based upon immunohistochemistry, AMH was localized primarily in the glandular epithelium and beneath the luminal epithelium. There did not appear to be differences in AMH expression between estrous and diestrous endometrial samples. RT-PCR revealed the expected 230 bp amplicon confirming that the equine endometrium expresses the gene for AMH. Although the role of AMH in the equine endometrium has not been defined, we speculate that AMH may regulate cellular proliferation or apoptosis in the endometrium possibly by acting in a paracrine/autocrine manner.

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Keywords: Equine, endometrium, anti-Müllerian hormone

Bioactivity of 5α-dihydroprogesterone in mares: endometrial response and maintenance of early pregnancy

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Objective: To investigate the progestagenic bioactivity of 5α-dihydroprogesterone (5α-DHP) on the endometrium and its ability to maintain early pregnancy in mares.

Materials and methods: In Exp. 1, ovariectomized mares (n=4) were treated daily with 5α-DHP (300 mg, im) or vehicle for ten days in a crossover design. Endometrial biopsies were taken immediately before the first administration of 5α-DHP and 24 hours after the last for routine histology and detection of the progesterone-responsive protein, lipocalin (P19). In Exp. 2, mares with confirmed Day 12 pregnancies (Day 0 = ovulation) were treated daily with 5α-DHP (0.7 mg/kg, im) (n=9) or vehicle (n=5), beginning on Day 13. On Day 14, mares were given PGF2α (10 mg Lutalyse® im;Pfizer Animal Health, New York, NY, USA) to eliminate endogenous progesterone. Plasma concentrations of progesterone and 5α-DHP were measured daily by liquid chromatography-mass spectrometry (LC-MS).

Results: In Exp. 1, 5α-DHP stimulated a progestagenic response in the endometrium characterized by increased glandular activity and by the presence of P19 which was not detected in vehicle-treated control mares. In Exp.2, conceptus development progressed to Day 27 (study endpoint) in seven of nine mares treated with 5α-DHP but in none of five control mares ($P<0.05$, Fisher's Exact test). Circulating concentrations of 5α-DHP maintained by exogenous administration were similar to the physiologic range of progesterone in cycling or early pregnant mares.

Conclusions: 5α-dihydroprogesterone is a bioactive progestagen capable of activating the endometrium, eliciting progesterone-responsive uterine secretion and maintaining early pregnancy in mares.

Acknowledgements

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Keywords: 5α-dihydroprogesterone, mare, pregnancy maintenance, progestin

Evaluation of an equine LH kit for prediction of ovulation

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Prediction of ovulation by measuring serum LH concentrations could be useful for timing insemination of mares when transrectal ultrasonography cannot be performed. Our objective was to evaluate the ability of an equine LH kit to predict ovulation in mares. Ovarian activity was monitored ultrasonographically, and serum was collected daily from six mares from the first day of behavioral estrus until 2 d post-ovulation. Serum samples from days -4 to 2 (Day 0 = ovulation) were analyzed in duplicates using an equine LH kit (Equine LH-Check®, Endocrine Technologies, Inc., Newark, CA, USA). Results (positive = serum LH concentration $\geq$ 2 ng/mL, or negative) were interpreted blindly by two operators. Agreement between operators, repeatability between duplicates, and day of first detection of a positive result were assessed. Positive and negative predictive value, sensitivity and specificity of the test for detecting ovulation within 24 h were calculated, and were 35, 94, 91.7 and 44.4%, respectively. Repeatability was moderate (74%) and agreement, high (97.4%). A positive result was first obtained 2.5 ± 1.2 d (mean ± SD) before ovulation, with all mares having high LH concentrations on days 0 to 2. While ovulation occurred in the face of high serum LH concentrations in all mares, a single positive result was not a reliable indicator of impending ovulation. However, a single negative result confirmed that ovulation had not occurred, or was not likely to occur within 24 h and breeding could be postponed.

Keywords: LH, ovulation, mare, prediction

Effect of short- versus long-proestrus on fertility in beef cattle after fixed-time artificial insemination

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The effect of changing the plasma progesterone concentration and duration of proestrus during growth of dominant follicle on fertility in beef cattle following fixed-time artificial insemination was determined. Heifers (n=61) and postpartum beef cows (n=80) were assigned randomly to four groups: high progesterone-long proestrus (HL), high progesterone-short proestrus (HS), low progesterone-long proestrus (LL) and low progesterone-short proestrus (LS). Cattle received two doses of prostaglandin F2α (PGF) 11 days apart, followed by estradiol-17β, progesterone along with a once-used CIDR device placed in vagina nine days later (Day 0). At the same time (Day 0), LL & LS groups were given two doses of PGF 12 h apart, while, HL & HS groups were allowed to retain the corpus luteum (CL). At CIDR removal on Day 7 (HL, LL) or Day 8 (HS, LS) cattle received PGF and pLH 12 h later to induce ovulation. Artificial insemination (AI) was done 12 h after the pLH injection. Low progesterone and long proestrus resulted in higher pregnancy rates ($P<0.023$; 54.0%, 38.2%, 46.4% and 12.2%, in LL, LS, HL and HS groups, respectively). There was no interaction between levels of progesterone and duration of proestrus ($P=0.12$) and the pattern was similar in cows and heifers ($P=0.47$). In conclusion, a short proestrous interval resulted in a reduced pregnancy rate following fixed-time AI in beef cattle. Although a low progesterone environment during growth of the ovulatory follicle increased ovulatory follicle size and subsequent CL size and function, it did not compensate for the effect of shortened proestrus on pregnancy rates.

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Keywords: Beef cows, heifers, fertility, proestrus, progesterone

Seminal parameters and field fertility of donkey semen cryopreserved in two different extenders

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Cryopreservation of jackass semen has been successfully performed using extenders commonly used to preserve stallion semen. However, differences in sperm physiology and/or plasma membrane composition could potentially result in different media requirements for cryopreservation of jackass semen. Our hypothesis was that jackass semen could be successfully cryopreserved in a simplified egg yolk-extender (Nagase).[1] The objective of the study was to compare seminal parameters and fertility of donkey sperm cryopreserved in Nagase versus lactose-EDTA extender.

Semen was obtained from five Pêga jackasses with histories of good fertility using fresh semen. In Experiment 1, five ejaculates from each male were collected using an artificial vagina, diluted 1:1 (v/v) with skim milk-based extender and centrifuged at 600 x g for 15 minutes. After centrifugation, the supernatant was discarded and the sperm pellet was re-suspended to 200 x 10^6 sperm/ml in Nagase or lactose-EDTA extender and loaded into 0.5 ml straws. Samples were cooled to 5 °C for 1 h and then placed over LN_2 (4 cm) for an additional 20 min, before being plunged into LN_2. Subjective evaluations were performed immediately after semen collection, after cooling to 5 °C and after freezing-thawing. Total motility (TM), progressive motility (PM) and plasma membrane integrity (HOST) were determined by a single person blinded to treatment groups. In Experiment II, semen from three males used in Experiment I and frozen in Nagase or lactose-EDTA extenders were used to inseminate 53 mares during a total of 60 cycles (n = 30 cycles per extender). Mares were inseminated within 6 h post-ovulation with $\geq$ 300 x 10^6 progressively motile sperm. Pregnancy diagnosis was performed by transrectal palpation and ultrasonography on days 15 and 25 post-ovulation. Seminal parameters were evaluated by ANOVA and individual means were compared by Tukey's test. Pregnancy rates were compared by chi-square. Statistical significance was set at $P<0.05$.

Seminal parameters were similar between groups either after cooling or after thawing (Table 1). However, cryopreservation significantly decreased total and progressive motility compared to cooled samples. Similar pregnancy rates were obtained when mares were inseminated with semen cryopreserved in Nagase or lactose-EDTA on days 15 [53% (16/30) vs. 50% (15/30)] and 25 [43% (13/30) vs. 47% (14/30)], respectively.

Table 1. Seminal parameters of donkey semen diluted in Nagase or lactose-EDTA extender and evaluated after cooling to 5 °C or after freezing-thawing (mean ± SD).

	Cooled Samples			Frozen-Thawed Samples		
	TM (%)	**PM (%)**	**HOST* (%)**	**TM (%)**	**PM (%)**	**HOST* (%)**
Nagase	80 ± 6	71 ± 7	51 ± 7	37 ± 4	32 ± 3	43 ± 5
Lactose-EDTA	78 ± 6	70 ± 6	45 ± 5	37 ± 6	31 ± 4	43 ± 5

* Percentage of cells with intact plasma membrane (positive HOST).

In summary, cryopreservation of jackass semen using simplified egg yolk-base extender (Nagase) resulted in seminal parameters and fertility similar to those of lactose-EDTA. Our results provide a practical and less costly alternative to cryopreserve donkey semen.

Acknowledgements

Financial support was provided by CNPq and Taruma Stud.

Keywords: Donkey, semen, cryopreservation, sperm, extender.

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Suppression of estrus in mares using a commercially available canine GnRH vaccine

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The objective of this study was to determine the effect of GnRH vaccination using a commercially available canine vaccine on cyclicity in the mare. The hypothesis was that this vaccine would induce GnRH antibody formation resulting in anestrus in previously cycling mares. Ovarian structures (follicles and corpora lutea) were monitored by transrectal palpation and ultrasonography three days per week. Jugular venous blood was collected from mares at time of palpation. After 4 weeks of initial monitoring, mares were divided into two groups, placebo-treated (n=5) and vaccinated (n=6). The vaccinated group received 5 mL IM (5x the canine dose) of Canine Gonadotropin Releasing Factor Immunotherapeutic® (Pfizer Animal Health, New York, NY, USA); whereas the placebo-treated group received an equal volume of sterile diluent provided by the vaccine manufacturer. In June 2008, initial intramuscular injections were administered into the semimembranosus muscle and not more than 2.5 mL of vaccine or diluent was injected at one site. Injections (placebo or vaccine) were repeated 3 weeks after the initial injection (July 2008). After each injection, mares were closely monitored daily for local and systemic vaccine reactions. None (0/11) of the vaccinated or placebo-treated mares developed any local or systemic adverse reactions following either the first or second injection. All placebo-treated mares (5/5) have continued to cycle normally through December 31, 2008. All GnRH vaccinated mares (6/6) stopped cycling (follicles <20 mm in diameter with no corpus luteum present) within 2 weeks after second vaccination. None (0/6) of the vaccinated mares had antibodies to GnRH prior to vaccination as determined by an enzyme linked immunosorbent assay (ELISA). GnRH antibody titers were detected in all (6/6) of the vaccinated mares by four weeks following the second injection. This protocol appears to be a safe and effective means of estus supression in mares. The reversibility of this protocol is currently under investigation as is the use of this method as a immunologic alternative to ovariectomy in embryo recipients.

Keywords: Anestrus, estrus supression, equine, GnRH, vaccine

The effect of geldanamycin on the cryosurvival of equine sperm

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The objective of this study was to evaluate the effect of geldanamycin, an inhibitor of heat shock protein 90, on the cryosurvival of equine sperm. Semen samples were obtained from five fertile stallions individually housed at UC Davis. One ejaculate from each of the stallions was collected using an artificial vagina. The sample was centrifuged at 300 g for 10 min. The pellet was resuspended in INRA 96 (IMV, Maple Grove, MN, USA), 2 % egg yolk and 0.3 M glycerol with differing concentrations of geldanamycin (0, 1.8, 4.5, 8.4 μM; Enzo Life Sci., Plymouth Meeting, PA, USA). Following cryopreservation one straw per treatment was thawed for 30 sec in a 37 ^{0}C water bath. Computer-assisted sperm analysis was used to determine sperm motility characteristics. Post-thaw membrane integrity was monitored with Sybr-14 and PI and fluorescence was measured using flow cytometry. Post thaw motility was measured at 5, 30 and 60 minutes. Treatment differences were calculated using a general linear model analysis of variance. The data were normally distributed, and post-hoc treatment comparisons were performed using Tukey's least squares method at a significance level of $P < 0.05$. There was no effect on post thaw viability (Mean=49.26% SEM=1.85) or 5 minute post thaw progressive motility (Mean=57.40% SEM=4.01) for any treatment group. There was a significant effect on 30 and 60 min progressive motility at 4.5 and 8.4 μM concentrations of geldanamycin. These results suggest a role for heat shock proteins under cryopreservation conditions in stallion spermatozoa.

Acknowledgements

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Keywords: Heat shock protein, geldanamycin, cryopreservation, equine, spermatozoa

Effect of intrauterine infusion of enrofloxacin on endometrial histology in mares

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Studies in vitro show that enrofloxacin is the only antibiotic to which most common endometritis-causing pathogens in mares have no resistance. Enrofloxacin could be a useful in the treatment of endometritis. Studies demonstrating the safety, tolerance, and side-effects of intrauterine infusion of enrofloxacin on the endometrium are scarce. Fumuso, et al. reported that intrauterine infusion of enrofloxacin in mares does not induce histological endometrial changes.[1] This is not consistent with field observations. The objective of this study was to determine if daily intrauterine infusion of enrofloxacin in mares causes significant acute and chronic endometrial inflammation and fibrosis.

Nine adult healthy mares with no clinical evidence of endometritis were used. Mares received daily intrauterine infusions of enrofloxacin (Baytril®100, 100 mg/ml, Bayer Animal Health, Shawnee, KS, USA) at 2.5 mg/kg for three days (note: off-label use of drug). Endometrial biopsies were taken prior to (S1) and at the end of treatment (S2) to evaluate acute effects of the antibiotic on endometrium. To evaluate the chronic effects, endometrial biopsies were taken at 14 days (S3) and 60 days post-treatment (S4). Biopsy samples were examined histologically and graded as described by Kenny and Doig (I, IIa, IIb, or III). Changes in endometrial biopsy grade within each mare were used to determine effects of treatment using a one-way ANOVA with correction for repeated measures.

Enrofloxacin induced acute epithelial ulceration, coagulative necrosis and hemorrhage of the stratum spongiosum which was evident in biopsy S2. The deeper endometrium had moderate to large amounts of pleocellular inflammation, edema, and hemorrhage. The S2 biopsies for all mares were categorized as endometrial grade III. In biopsy S3 most mares had evidence of significant fibrosis and inflammation consistent with grade IIb or III. In biopsy S4, fibrosis was extensive with variable inflammation, consistent with grade IIb or III; an overall worsening of endometrial biopsy score by 1 to 3 grades.

The cause of endometrial lesions could be the high pH (10.4) of enrofloxacin and/or biochemical alteration of proteoglycans within vascular endothelium. Enrofloxacin inhibits proliferation of equine tendon cells due to impaired proteoglycan synthesis. In laboratory animals and women, proteoglycans within the endometrial epithelial cells have been shown to contribute to a non-thrombogenic surface on vascular endothelium.

Keywords: Mare, enrofloxacin, intrauterine infusion, uterine biopsy

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Kisspeptin-induced LH response in diestrous and anestrous mares

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Recently demonstrated to be a key player in many reproductive processes, kisspeptin has been shown to control the timing and release of GnRH and move anestrous ewes to ovulation. Our goals for this study were to test the hypotheses that mares would respond to an iv dose of this peptide with a subsequent rise in plasma LH, and that an infusion of this peptide would induce ovulation or luteinization of follicles in seasonally acyclic mares.

Six light horse mares in mid-diestrus were used in a cross-over design with three groups of two mares each and treated with iv saline or kisspeptin (1.0 or 0.5 nmol/kg; KP-10, human metastin 45-54; Peptide Institute Inc., Osaka, Japan). Mares responded to the kisspeptin treatment with a rise in LH concentration ($P<0.05$), and both doses achieved a similar response. Due to the equivocal response, the protocol was repeated with KP-10 doses which were lower than previously tested (0.10 or 0.05 nmol/kg), with both lower-dose treatments exhibiting inconsistent results. To determine whether kisspeptin could induce ovulation in seasonally acyclic mares, two groups of mares (n=3 per group) were infused for 20 hours with either saline or KP-10 [100 μg/hr iv (77 nmol/hr)]. There was no effect of kisspeptin on plasma LH in acyclic mares, and no evidence of ovulation or luteinization of follicles as confirmed by plasma progesterone levels <1.0 ng/ml from Days 0 to 5 post-infusion.

In contrast to the seasonality work performed in ewes, these data suggest that acyclic mares may not be capable of responding to kisspeptin. To test this hypothesis, a dose of KP-10 previously demonstrated to induce a reproducible response in the diestrous mare (0.5 nmol/kg) or saline was administered as a single iv bolus to five seasonally acyclic mares in a cross-over design. Acyclic mares responded to KP-10 with a rise in plasma LH ($P<0.05$), but this response was considered physiologically insignificant when compared to the robust response in the diestrous mare during the natural breeding season. These data suggest a regulatory role for kisspeptin in cyclicity and seasonality of mares, but imply that mechanisms in long-day breeders like the mare differ from those in short-day breeders like the ewe. There is evidence that this neuropeptide may be a central coordinator responsible for translation of necessary signals such as photoperiod, nutritional and hormonal status in order to induce GnRH release and initiate cyclicity. However, this pathway for signal transduction in the mare remains to be elucidated.

Keywords: Kisspeptin, equine, LH, ovulation, seasonality

Toll-like receptor 4 tissue expression in the equine endometrium

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The objective of this study was to determine the levels of Toll-like receptor 4 (TLR-4) tissue expression in the equine endometrium, and the relative levels of TLR-4 tissue expression in mares resistant and susceptible to endometritis. All mares were evaluated for susceptibility to endometritis using the standard *Streptococcus equi* subspecies *zooepidemicus (Strep)* challenge model. Briefly, clean mares were infected with 10^5 colony forming units of *Strep* on the day of ovulation then evaluated for uterine inflammation (uterine culture and cytology) at 72 hours. Mares with no infection/inflammation at 72 hours were considered resistant (R, n=5); those with significant uterine bacterial growth and/or more than 5% neutrophils on cytology were considered susceptible (S, n=4). At the 72-hour post-challenge evaluation a uterine biopsy was also taken. After treatment of all infected mares (uterine lavage and antibiotics as needed), each mare received at least one untreated cycle and then in a subsequent uninfected estrous cycle an endometrial biopsy was performed within 24 hours of ovulation. Endometrial tissues were frozen in liquid nitrogen and stored until further processing. Endometrial tissues were thawed at room temperature, lysed, and mRNA was extracted (QIAshredder, RNeasy Mini kit, Qiagen, Inc., Mississauga, ON, Canada). The mRNA was processed into cDNA and real-time polymerase chain reaction (RT-PCR) was performed for each sample in duplicate (QuantiTect Reverse Transcription Kit, Qiagen; Brilliant SYBR Green QPCR Master Mix, VWR International, LLC, Edmonton, AB, Canada). The primers for TLR-4 and glyceraldehyde-3-phosphate dehydrogenase (normalizing gene, GAPDH) had been previously validated (custom primers, Invitrogen Canada, Inc., Burlington ON, Canada)[1]. The mRNA concentrations were measured by evaluating the TLR-4 PCR cycle threshold (Ct) then standardized to the GAPDH Ct, resulting in the adjusted Ct (ΔCt). The difference between the post-*Strep* level and the estrous level of mRNA was calculated (ΔΔCt). The fold-change in the mRNA level was calculated using the equation $2^{-\Delta\Delta Ct}$. The paired t-test was used for analysis of differences of the ΔCt at estrus and post-*Strep* in the mares by category and as a single group. A two-sample t-test was used to compare the ΔΔCt and the fold-change differences between R and S mares (Stata 10.0, StataCorp, College Station, TX, USA). No differences were found between R and S mares in terms of the ΔCt, ΔΔCt, or $2^{-\Delta\Delta Ct}$. Values of the ΔCt of the combined groups of mares post-*Strep* (2.4 ± 0.5, mean ± SD) tended to be increased relative to values at estrus (1.5 ± 1.8) ($P < 0.09$). These findings suggest a different level of TLR-4 tissue expression in the endometrium of mares in estrus compared to post *Strep* infection. No differences in TLR-4 tissue expression were found between R and S mares that could account for their differing responses to intrauterine infection with *Strep*. The lack of significant differences may be due to the small sample size in this study. This is the first report of TLR-4 tissue expression in the equine endometrium demonstrated through real-time PCR.

Keywords: Toll-like receptor-4, endometritis, real-time PCR, RNA, mare

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Evaluation of pathogen progression during induced placentitis in mares using *lux*-modified *Escherichia coli* and novel bioluminescence imaging technology

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Placental infection due to opportunistic pathogens such as *Escherichia coli* (*E. coli*) is a common cause of abortion, stillbirth and premature delivery in horses. Moreover, pathogen progression during placentitis may involve invasion of fetal tissues, including the brain, leading to increased pro-inflammatory cytokine expression resulting in onset of premature delivery and/or fetal neurological damage. Thus, the objective of this pilot study was to monitor pattern of pathogen progression and invasion of fetal tissues by experimentally-inducing uterine infection of mares with a *lux* gene-modified *E. coli* using real time bioluminescence (biophotonics) imaging technology. To this end, one horse (~280 d gestation) and two pony (~300 d) mares were inoculated trans-abdominally (ultrasound-guided intra-amnion) with 2 x 10^6 colony forming units of *E. coli* (CFU in1 mL of broth) transformed with the pAK1-*lux* plasmid (*E. coli-lux*). The plasmid (11,904 bp) used is a broad-host-range cloning vector with numerous plasmid replicons. Trans-abdominal and –rectal ultrasonography was performed every 12 h for confirmation of fetal viability. One pony mare and the horse mare aborted ~24 h post-inoculation while the third fetus was recovered at 40 h post-infection following euthanasia of the mare. Fetuses recovered immediately post abortion and the intact uterus of the third mare were subjected to biophotonic imaging using a NightOwl imaging system (Peltier cooled slow scan CCD camera; Berthold Technologies, Oak Ridge, TN, USA) for detection of *lux*-expressing (photon emission) bacteria. Scans were performed over a 5 min period to accumulate photons indicative of pathogen presence in localized tissues. Subsequent to intact uteri and/or whole fetus imaging, fetuses were dissected and heart, lungs, liver, bladder, gastro-intestinal (GI) tract and brain were removed and imaged. *Lux* emitting bacteria were found in the lungs, GI tract, nares and sinuses but not in the brain, heart or liver in the two fetuses recovered at 24 h post-inoculation. Fetal amniotic, GI tract, stomach, bladder, and pericardial fluids were analyzed for presence of emitting bacteria and to determine total bacteria counts. Cultures of amniotic, stomach and bladder fluids confirmed presence of *lux*-emitting bacteria with counts ranging from 10 x 10^6 to 140 x 10^6 CFU/mL for amniotic and GI tract fluid, respectively, but no counts in pericardial fluid. Histopathology confirmed bacterial colonization of the fetal brain at 280 d but not at 300 d, which may suggest differences in stage of cerebral development. No *E. coli-lux* emitting bacteria were identified in the fetus or fetal fluids recovered from the mare at 40 h. These data demonstrate that bioluminescence and real time imaging provide a novel means of understanding pathogenesis of bacterial-induced placentitis and preterm birth in horses. The application of this novel imaging technology with *lux*-modified organisms may facilitate the development of more targeted therapeutic interventions.

Acknowledgements

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Keywords: Placentitis, pathogenesis, foal, *E. coli-lux*, bioluminescence imaging

Preliminary evidence of fetal hypothalamic-pituitary-adrenal axis activation in an experimental model of infective preterm delivery in the mare

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Activation of the hypothalamic-pituitary-adrenal axis (HPAA) is a key event in the control of labor at term, and immaturity of the HPAA at birth is a major factor leading to poor neonatal outcome with preterm delivery. Cortisol production in the fetal horse rises only immediately prior to term gestation; manipulations to accelerate fetal maturation precociously are frequently complicated with a negative neonatal outcome. Previous research suggests that the HPAA in equine fetuses less than 295 d are immature, as evidenced by failure of intrafetal ACTH administration to elicit cortisol production by the fetus. Women with intrauterine infection and preterm delivery had significantly higher amniotic fluid concentrations of cortisol than patients with preterm delivery without intrauterine infection. Changes in equine fetal plasma cortisol concentration in response to *in utero* infection have not previously been reported, nor have concentrations of cortisol in equine fetal fluid. The objectives of this study were to measure changes in cytokine expression in the chorioallantois and cortisol concentrations in fetal fluid from mares in an experimental model of infective pre-term delivery. Thirteen adult pony mares of various ages were used in this study over a 2-year period. Allantoic catheters were placed in sedated standing animals under local anesthesia using laparoscopic visualization. Seven mares had *in utero* infection, six mares were uninfected. Fetal fluid and maternal plasma was collected at twenty-four hour intervals, centrifuged, and the supernatant stored at -70 °C. The concentration of cortisol in fetal fluid was assessed using commercially available radioimmunoassay reagents (Cortisol RIA, Diagnostic Systems Laboratories, Inc., Webster, TX, USA), previously validated for equine samples. Samples were assayed in duplicate. Four areas of the chorioallantois were collected at delivery and stored in RNA stabilization reagent (RNAlater, Qiagen Inc., Valencia, CA, USA), until analyzed for expression of a panel of eleven equine-specific cytokines. Experimentally-induced infection increased the expression of IL-1β, IL-18, IL-15, IFN-γ, in a site dependant manner. Mares spontaneously aborting also had increased expression of IL-1β, IL-18, IFN-γ, and iNOS in a site dependant manner. Data from eleven mares were included for fetal fluid cortisol analysis; five mares had *in utero* infection, six mares were uninfected. Substantial increases in cortisol concentration in fetal fluids were observed prior to spontaneous abortion in three mares (two with fetal infection and placentitis, one with fetal aseptic fibrinous pneumonia and placental edema). Maternal plasma cortisol concentrations are pending. These results suggest that increased cortisol concentration in fetal fluid may be seen with infection or inflammation at 80% gestation. The signaling pathways responsible for release of cortisol from the equine fetal adrenal gland subsequent to intrauterine infection are unknown, but data from other species would suggest that exposure to pro-inflammatory cytokines is one likely mechanism resulting in fetal HPAA activation. All of the mares with increased fetal fluid cortisol concentrations had a greater than 25-fold expression change in IL-1β at the cervical star, and in normal and abnormal areas of the chorioallantois. Further investigations, such as *in vitro* fetal adrenal cell responsiveness to IL-1β and ACTH at various stages of gestation may provide insights into the signaling pathways leading to cortisol secretion subsequent to IL-1β treatment or exposure.

Keywords: Equine, fetus, hypothalamic-pituitary-adrenal axis, pre-term delivery, inflammation

Experimentally induced placentitis in late gestation mares with *Streptococcus equi zooepidemicus*: therapeutic prevention of preterm birth

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Placental infection due to opportunistic pathogens (i.e., *Streptococcus equi* subspecies *zooepidemicus (S. equi))* is the single most common cause of abortion, stillbirth and premature delivery in horses. Recent evidence suggests that placentitis increases proinflammatory cytokine expression leading to premature delivery. The objective of this study was to evaluate the efficacy of using antibiotics alone or in combination with immunomodulators to prevent preterm birth and increase foal viability in mares experimentally infected to induce ascending placentitis.

Twenty three pregnant (299.4 ± 1.7 d; light breed) mares were assigned to one of four treatments. Seventeen were infected (298 ± 1.6 d) by intra-cervical inoculation with *Streptococcus equi zooepidemicus* (~$2x10^6$ CFU) and assigned to receive trimethoprim sulfamethoxazole (TMS; 30 mg/kg, q 12 h, PO, n = 6) alone; or with either dexamethasone (D) given over 6 d with decreasing doses every two d (TMS+D; 40, 35, 25 mg, respectively, q 24 h, IV, n = 6) or acetylsalicylic acid (TMS+A; 50 mg/kg, q 12 h, PO for 6 d, n = 5). Six mares served as uninfected controls (CN). Blood samples were collected pre- and post-infection at 12, 24, 48, 72 h and 3x/week thereafter until delivery for progesterone (P4) analysis. Fetal and placental well-being was evaluated daily by ultrasonography. Treatment commenced upon signs of vaginal discharge and/or placental changes. Blood was collected from foals at 0 and 24 h post-partum for CBC, IgG and P4. Inoculation induced vaginal discharge within 48 h and increased ($P<0.05$) combined thickness of uterus and placenta in all mares (0.83 ± 0.03 to 1.5 ± 0.1 cm). Mean serum P4 was not affected by inoculation or drug treatment, but P4 concentrations in foals of infected dams ranged from 4.6 to 24.5 and 0.9 to 15.3 ng/mL, and from 1.8 to 9.9 and 0.5 to 1.8 ng/mL in foals of control dams at 0 and 24 h, respectively. Mean gestational age at term was less (307.6 ± 3.7 d; $P < 0.05$) in TMS+D mares than in the other groups (CN, 338 ± 5.0; TMS, 318.8 ± 5.0; TMS+A, 322.6 ± 6.6 d). Birth weights were lower in TMS+D foals (36. 8 ± 1.7 kg; $P <0.05$) compared to foals of mares in the other treatment groups (CN, 45.5 ± 4.0; TMS, 44.6 ± 2.9; TMS+A, 45.2 ± 3.0 kg), but birth weight-placental weight ratio was higher ($P <0.05$) in all infected mares compared to controls. Placental pathology confirmed necrosuppurative placentitis in all inoculated mares. Mares delivered six viable foals in the CN group, four in each of the infected groups, two aborted in the TMS group, one live (euthanized at 24 h) and one aborted in the TMS+D group and one aborted in the TMS+A group. In these studies, four of the six (67%) mares treated with antibiotics alone had successful pregnancy outcomes compared with 8 of 11 (73%) mares treated with the drug combination, suggesting that aggressive therapy with antibiotics can substantially improve pregnancy outcome.

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Keywords: Mare, placentitis, antibiotics, immunomodulators

Pharmacokinetics of a single injection of progesterone in oil, and factors affecting serum progesterone analysis in alpacas

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Progesterone concentration (P4) in serum or plasma is used commonly to determine pregnancy and cyclicity status in alpacas. Diagnostic errors have been relatively frequent in cases referred to our clinic. Therefore, we determined if sample handling factors and laboratories affect progesterone concentration in submitted samples.

Six adult non-mated female alpacas received a luteolytic dose of cloprostenol (250 μg, IM) 24 hours prior to sampling, and were examined by ultrasonography to eliminate any endogenous source of P4. Each female received a single IM injection of 25 mg progesterone in oil. Blood samples were taken for P4 determination at 0, 1, 2, 4, 6, 8, 10, 12, 18 and 24 hours following treatment. Sample handling factors included type of anticoagulant (none, sodium heparin, EDTA), and storage time in separator tubes (48 vs 72 hours) or red top tubes (0, 48 hours and five days) at 5 °C. All samples were centrifuged after collection and storage, and stored at -20 °C until analysis. Split samples from the eight-hour sampling time were submitted to two commercial laboratories. All other assays were done at the WSU endocrinology laboratory (J.J. Reeves) by radioimmunoassay. Plasma or serum P4 assays were done using a general linear model for sample handling and laboratory comparisons.

Serum P4 increased rapidly after injection, reached a peak between four and six hours, then decreased steadily from 10 hours. There was an individual female effect ($P < 0.05$) on the area-under-curve analysis and slope of decrease in P4 following injection.

None of the sample handling factors had a significant effect on P4 concentration. However, the laboratory had a significant effect on P4 levels. Agreement among laboratories was 100% for samples known to be positive for P4 (>1.5 ng/mL). However, one laboratory reported high (> 1.5 ng/mL) and suspect (>1 and <1.5 ng/mL) P4 in 13.3% and 26.6 % of the negative (P4 < 0.5ng/ mL) samples, respectively.

We conclude that a single injection of P4 in oil results in a concentration of P4 compatible with pregnancy for at least 24 hours. Response varies with individual animals, which is mostly likely due to body weight and metabolic difference. No effect of anticoagulant was detected, and samples taken in red top tubes can be stored at 5 °C for up to five days without a detectable effect. The most important factor in clinical measurement of P4 is the reliability of the laboratory.

Keywords: Camelid, progesterone, pregnancy diagnosis

Pregnancy rate following in vitro culture conditions of and transfer of hatched dromedary camel (*Camelus dromedarius*) blastocysts

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Efficiency of embryo transfer programs in the dromedary are often handicapped by the unavailability of synchronized recipients. In the absence of adequate techniques for cryopreservation of large dromedary blastocyst, other alternatives for short term preservation need to be developed. The aim of this study was to evaluate the effect of two in vitro culture (IVC) conditions on the quality of camel embryos and their viability after embryo transfer. Hatched blastocysts (n=46) were collected from stimulated females eight days after mating by uterine flushing using modified Dubelcco's phosphate buffer saline. Group 1 embryos (n= 24) were co-cultured at 20% of O_2 with camel epithelial oviductal cells in presence of M199 + 10% heat treated fetal calf serum (htFCS) for two (n= 12) or four days (n= 12). Group 2 embryos (n= 22) were cultured in KSOM medium supplemented with 10% heat-treated fetal calf serum at 5% of O_2 for two (n= 12) or four days (n= 10). All in vitro cultures were done at 38.5 °C and maximum humidity (>95%).

After the period of culture, all embryos were transferred individually to synchronous recipients. Pregnancy rates following transfer (Table 1) were significantly better for short term (two days) co-culture system than all other treatment groups. Pregnancy loss between two and six months following transfer was significantly higher for embryos cultured in defined medium (KSOM) and for embryos in co-culture with epithelial oviductal cells for four days. The only pregnancies that survived to term and have given birth thus far were obtained from embryos co-cultured with oviduct cells for two days. These preliminary results show that camel blastocyst can be preserved for two days by co-culture with oviductal cells and achieve pregnancy rates at 60 days following transfer similar to fresh embryos in our laboratory. However, pregnancy loss between 60 days and 180 days is significantly higher than we usually experience with transfer of fresh embryos.

Table 1. Pregnancy rate following transfer of hatched dromedary blastocysts after culture for two or four days in co-culture with oviductal cells (Group 1) or KSOM (Group 2).

Treatment group	Embryos transferred	Pregnant at 60 days (%)	Pregnant at > 6 months (%)
Group 1, 2 days	12	11 (92)[a]	8 (67)[a]
Group1, 4 days	12	7 (58)[b]	0 (0)[b]
Group 2, 2 days	12	5 (42)[b]	2 (17)[b]
Group 2, 4 days	10	0[c]	0[b]

Values in the same column with different superscripts differ significantly (P<0.05). *To date, four (50%) have delivered calves.

Keywords: Preservation, culture, embryo, pregnancy, viability

Imipramine and xylazine treatment does not induce ejaculation in alpacas

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In stallions, semen can be collected without copulation (*ex copula*) through the means of pharmacologically-induced ejaculation with imipramine and xylazine.[1,2] A preliminary study in llamas revealed that imipramine and xylazine treatment induced ejaculation in 80% of attempts.[3] The objective of the present study was to determine if imipramine and xylazine treatment would induce ejaculation in alpacas. Intact male alpacas (n=11) were used with ages ranging from 2-10 years (6.5±2.6 years). Two males had sired crias, seven males had never been used for breeding and the reproductive history of two males was not known. Animals were sheared and body weights were determined. Right jugular venous catheters were placed to facilitate imipramine and xylazine administration. A Whirl-pak® (Nasco, Ft. Atkinson, WI, USA) bag was taped over the preputial opening prior to each treatment and removed 10-20 minutes following treatment. Seven treatment protocols were evaluated: (1) saline (control); (2) only xylazine (0.1 mg/kg); (3) only xylazine (0.2 mg/kg); (4) only imipramine (1.0 mg/kg); (5) imipramine (1.0 mg/kg) followed ten minutes later with xylazine (0.1 mg/kg); (6) imipramine (2.0 mg/kg) followed ten minutes later with xylazine (0.1 mg/kg); and (7) imipramine (1.0 mg/kg) followed twenty minutes later with xylazine (0.1 mg/kg). Each treatment protocol was repeated two or three times in each animal. For small volume (<0.5 mL) samples collected within the Whirl-pak® bag after each treatment, impression smears were made on glass microscope slides and the presence of spermatozoa was determined following Diff Quik® (Siemens Healthcare Diagnostics, Deerfield, IL, USA) staining. When urination occurred, samples were centrifuged and Diff-Quick® stained sediment samples were evaluated microscopically. Preputial secretions (pH ranged from 7 – 9) were present from at least one male following each treatment (including control) except for the imipramine (1.0 mg/kg) and xylazine (0.1 mg/kg) protocol in which one of the three replications did not yield preputial secretions in any of the males. No spermatozoa were in any of the samples containing preputial secretions. Few spermatozoa were present in two urine sediment samples from one male following two of the three treatment replications with imipramine (1.0 mg/kg) and xylazine (0.1 mg/kg). In another male, few spermatozoa were present in one urine sediment sample following one of the three treatment replications with imipramine (1.0 mg/kg) and xylazine (0.1 mg/kg) and following one of the two treatment replications with only xylazine (0.2 mg/kg). Spermatozoa were not present in urine sediment samples from these males following other treatment protocols. It is not clear why *ex copula* ejaculation could not be induced in these sexually mature alpacas but may have resulted from differences in drug compounding of imipramine between the two camelid studies.

Keywords: Alpaca; *ex copula* ejaculation; imipramine; spermatozoa; xylazine

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Production of hybrid (Bighorn x domestic sheep) lambs by laparoscopic artificial insemination using Bighorn fresh semen collected by electroejaculation

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Bighorn sheep are extremely susceptible to pneumonia when they are in contact with domestic sheep and death rate is very high. Hybrid animals can provide an excellent research model for comparative immunology studies. Although there are anecdotal reports of hybrid domestic sheep (*O. aries*) and Bighorn rams (*Ovis canadensis canadensis*) offspring, no information is available on specific conditions of breeding. We hypothesized that production of hybrid lambs can be obtained by laparoscopic artificial insemination of domestic sheep using fresh semen from Bighorn rams. Our objectives were to investigate semen collection and short term preservation of semen from Bighorn rams, to determine the success rate (pregnancy and lambing rates) of laparoscopic insemination (LAI) of domestic ewes with Bighorn ram semen and gather biological data on hybrid pregnancies and lambs.

Semen was collected by electroejaculation from Bighorn rams anesthetized with tiletamine and zolazapam (Telazol®, 4.4 mg/kg, Ft. Dodge Animal Health, Ft. Dodge, IA, USA) and xylazine (2.2. mg/kg). Insemination was performed on 19 ewe-lambs synchronized with home-made vaginal pessaries containing progesterone inserted for 12 days. All ewes received an IM injection of a dose of PG 600® (Intervet/Schering-Plough Animal Health, Millsboro, DE, USA)) and cloprostenol (250 μg) when the pessaries were reomoved and were inseminated laparoscopically (LAI) with approximately 50 million progressively motile spermatozoa under general anesthesia 60 hours later. Pregnancy diagnosis was performed by ultrasonography 42 days after insemination.

Semen was successfully collected in five out of six attempts during the peak of the breeding season and all samples showed >80% progressive motility and >90% normal morphology and survived chilling in commercial ovine semen extender for four days (progressive motility >50%). LAI resulted in five pregnancies but two ewes lost their pregnancy between 42 and 60 days. The remaining three ewes lambed (two, one and two lambs) after a pregnancy length of 147, 148 and 146 days, respectively. Lambs weight varied from 1.2 and 1.75 kg. The placentae were smaller and had fewer cotyledons than normal. These preliminary results show that semen can reliably be collected from Bighorn rams under general anesthesia and be preserved for at least four days at 5 °C using commercial ovine semen extender. The pregnancy and lambing rate in this trial were low compared to expected results in domestic sheep. More studies are needed to determine the effect of these hybrid pregnancies on placental function and fetal development.

Keywords: Insemination, placenta, lambs, hybrids

Teaching veterinary obstetrics using three-dimensional animation technology

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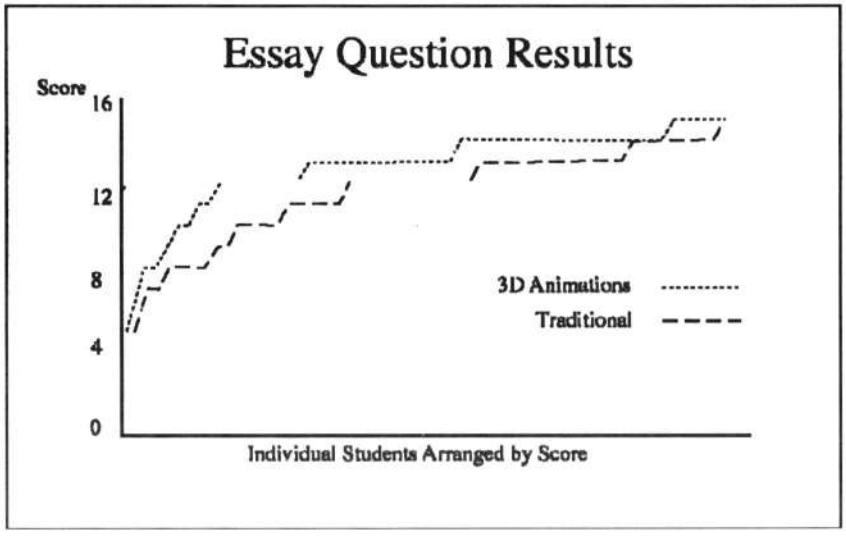
Historically, the teaching of veterinary obstetrics has been limited to descriptions of birth and dystocia through the use of photographs, text, and two-dimensional graphical presentations.

We hypothesize that the use of three-dimensional, interactive, digital animations will help students visualize, and therefore better comprehend the complex processes during both normal birth and dystocia. The *Veterinary Obstetrics Project* is developing both animations and interactive QuickTime VRs for use in instruction. The animations demonstrate normal birth, as well as dystocias and their appropriate treatments. The QuickTime VRs allow the students to interact with the three-dimensional model and examine the cow and calf from all angles.

In this two-year study, second-year veterinary students were taught using traditional materials in 2007 (n=62), and with the three-dimensional materials in 2008 (n=60). In addition to multiple-choice questions (maximum score of 80 points), the final examination in both years included essay questions designed to assess students' comprehension of normal and abnormal presentations of the calf (maximum score of 15 points). Data were analyzed with the Mann-Whitney U test using Minitab statistical software.

Student scores for the multiple-choice questions were not different between years (medians 75 in 2007 and 74 in 2008). In contrast, student scores for the essay questions were significantly different between years (P = 0.001).



The results of this study indicate that the incorporation of three-dimensional animations of normal parturition and dystocia into the course enhanced students' understanding of these processes.

Keywords: Three-dimensional animation technology, veterinary obstetrics, teaching.

Identifying gross chromosomal rearrangements that result in infertility in camelids

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We have conducted data mining in the archives of cytogenetic service laboratories in U.S. and discovered that alpacas and llamas display a variety of chromosomal rearrangements that are associated with impaired fertility. Among these, the presence of one abnormally small ("minute") chromosome characterizes almost 28% of all infertility cases submitted for cytogenetic analysis in camelids. During the past six years, our research group has identified seven female alpacas with the "minute" chromosome. All animals were infertile with phenotypic features such as very small ovaries (<0.5 cm in diameter) with no follicular development and an underdeveloped uterus. A typical karyotype containing the "minute" chromosome (74,XX minute) is shown in Fig. 1A. Though several hypotheses have been proposed regarding the origin of the "minute", the nature of the rearrangement remains unresolved. Besides this, we have identified other types of chromosomal rearrangements in infertile individuals. For example, we discovered an autosomal translocation in a male llama (Fig. 1B) that had normal breeding behavior and was normal on physical examination, but he had never produced any crias despite breeding multiple females over two years and had nearly 100% abnormal spermatozoal morphology. Due to the complexity of camelid karyotypes (high diploid number and similar morphology between different chromosome pairs), it is not possible to unambiguously identify the chromosomes solely based on traditional cytogenetic approaches. Development of molecular markers for chromosomes involved in rearrangements is therefore necessary. In preliminary studies, we have used fluorescent *in situ* hybridization (FISH) with selected alpaca flow sorted chromosome paints. We have identified a composite probe which, in addition to other chromosomes, contains the DNA sequences corresponding to the "minute". Similarly, using X- and Y-specific chromosome paints, we have confirmed that the translocation in male llamas is autosomal and does not involve sex chromosomes.

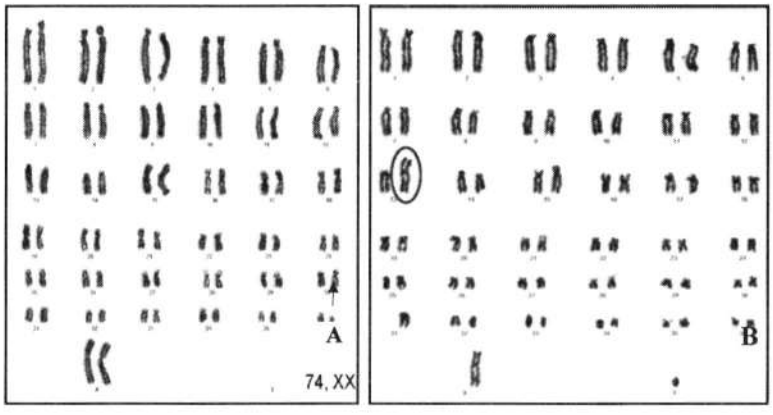

Fig.1. Karyotypes from a 74,XX female alpaca (A) with the "minute" chromosome (arrow) and a 73,XY male llama (B) with an autosomal translocation (circle).

Comparison of sperm morphology and DNA fragmentation in normal and asthenozoospermic dogs

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Sperm motility and morphology have been correlated in various studies with fertility. Recently, the influence of sperm DNA fragmentation on fertility has been investigated. The objective of this study was to compare sperm morphology abnormalities and DNA fragmentation in normal (≥80% progressively motile sperm; n=4) and asthenozoospermic (≤60% progressively motile sperm; n=6) dogs. Seven breeds of dogs were represented. Mean age per group was not significantly different. Semen ($1^{st}/2^{nd}$ fraction) was collected by manual stimulation in the presence of an anestrous teaser. Sperm were evaluated under light microscopy for total motility, progressive motility and speed. Sperm concentration was determined using a hemocytometer. Sperm morphology was determined following staining with eosin and nigrosin and one individual (HM) counted 200 cells under oil immersion at 1000X magnification. In addition, a semen sample (1 mL) from each dog was frozen and shipped on dry ice for sperm chromatin structure analysis (SCSA).[1] Briefly, aliquots (2-7 µl) of thawed semen samples were diluted to 200 µl in a Tris buffer solution and mixed with 400 µl of acid-detergent solution. A DNA probe (acridine orange; 1.2 ml) was added to each sample. Using a flow cytometer, 5000 spermatozoa/sample were analyzed at a flow rate of 100-200 cell/sec. The percentage of spermatozoa with altered chromatin structure was identified as those with increased red fluorescence corresponding to increased DNA fragmentation (DFI).[1] Half (3/6) of the asthenozoospermic dogs had a DNA fragmentation index (DFI) >30% whereas none (0/4) of the dogs with normal sperm motility had a DFI >30%. However, with respect to sperm morphology, only dogs with ≥10% head abnormalities had a DFI >30%. In addition, there was a significant positive linear correlation between percent sperm head abnormalities and %DFI. In other species, a DFI >30% is statistically correlated with a decrease in term pregnancies.[1] However, it is important to note that a DFI value above 30% does not preclude a normal, full-term pregnancy. Based upon these findings, sperm morphology, specifically head abnormalities, was correlated with poor fertility and increased % DFI, whereas sperm motility was not. Due to the small sample size in this study, further research is needed to corroborate these findings.

Keywords: Asthenozoospermia, DNA, dog, SCSA, sperm

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Accuracy of canine parturition date prediction from LH peak

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Accurate prediction of parturition is valuable in managing canine pregnancy and parturition. The gestation length of the dog varies considerably depending on how it is defined. Gestation can be measured based on breeding dates, cytologic estrus and diestrus based on vaginal cornification, date of ovulation (which cannot be measured by standard methods), or dates of hormonal changes, most commonly progesterone (P4) and luteinizing hormone (LH).

We sought to determine by retrospective analysis if using a combination of pre-breeding progesterone and LH levels would narrow the window and increase the accuracy of prediction of parturition date using the Witness® LH test (Synbiotics, Kansas City, MO, USA) a rapid immunomigration (RIM) test.

Serial serum samples were collected from 66 bitches (consisting of four breeds including Labrador retriever, golden retriever, German shepherd, and Labrador-golden crosses) for a total of 98 ovulation cycles. P4 levels were measured on samples every other day by radioimmunoassay (RIA) or chemiluminescent immunoassay (CLIA) through the Cornell University Diagnostic Laboratory. An in-house Witness® LH test was performed on saved serum samples from the date when P4 rose to $\geq$ 1.5 ng/ml and was at least twice the baseline progesterone level. Day 0 (d0) was defined as the day serum tested positive for LH on the in-house LH test. The average concentration of P4 on the day of the LH surge was 1.97 ± 0.66 ng/ml. Dogs were bred on d3 and d5 or d4 and d6. The predicted parturition date, 65 days following the day of the LH rise (d65), was compared to actual parturition date, the day the first pup was delivered. We determined that the accuracy of parturition date prediction within a ± 1 and ± 2 day interval was 82 and 100%, respectively and that accuracy was not affected by breed or litter size. A previous study found an accuracy of parturition prediction within ±1, ±2 and ±3 days using prebreeding serum P4 alone to be 67, 90, and 100%, respectively.[1]

Keywords: Canine, LH surge, pregnancy, parturition, progesterone

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Pharmacokinetics and ovarian stimulatory effects of eCG in the bitch

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Two experiments were designed to investigate the pharmacokinetics and ovarian stimulatory effects of eCG in the bitch. Experiment 1 studied the area under the curve (AUC) of eCG iv and im. Six adult, mixed breed neutered bitches aged between 2 and 5 yr and weighing between 15 and 20 kg were used. Animals were divided into two groups. Group I received 10.000 IU of eCG iv and group II received 10.000 IU of eCG im. Blood samples were taken 25 min before eCG administration, 30 min, 1, 2, 3, 6, 9, 12, 18, 24 h after eCG administration and daily for next 7 days. All samples were centrifuged and stored at -20 °C until eCG concentrations were measured using a validated enzyme immunoassay in unextracted bitch serum. The AUC was calculated using Sigma Plot® 8.02 (Systat Software, Inc., San Jose, CA, USA). Experiment 2 studied the clinical and cytological effect of several doses of eCG. Twenty five adult, mixed breed, intact bitches aged between 2 and 7 yr and weighing between 10 and 27 kg were used in a randomized design. During late anestrus, bitches were assigned to one of five different treatments: TRT1, 5 IU/kg eCG im (Novormon®, Syntex SA, Argentina; n=5); TRT2, 10 IU/kg eCG im (n=5); TRT3, 20 IU/kg eCG im (n=5); TRT4, 44 IU/kg eCG im (n=5); and TRT5, 50 IU/kg eCG im (n=5). Estrus behavior (score 1-3) was recorded and vaginal cytology samples (score 1-5) were obtained before eCG administration and every other day during 15 days. Blood samples were taken before eCG administration to measure P_4 to confirm anestrus. All blood samples were centrifuged and stored at -20 °C until P_4 was measured by solid RIA. Data were analyzed by ANOVA. Data are represented as least square means ± SEM. Significance was defined as $P < 0.05$. In experiment 1, there were no statistical differences in the AUC in bitches treated im or iv (311.51 vs. 254.81±36.91 IU h/ml, $P<0.33$). In experiment 2, bitches from TRT5 had a significantly higher vaginal cytology scores and estrus behavior scores compared to bitches from TRT1-4 after eCG treatment (5.00±0.46 vs. 2.21±0.21, $P<0.0001$; 3.00±0.11 vs. 1.08±0.05, $P<0.0001$; respectively). Conversely, P_4 concentrations before eCG administration were similar in all TRT groups (0.26±0.06, $P<0.72$). In conclusion a single administration of 50 IU/kg of eCG im could be used to induce follicular development in the bitch.

Keywords: Bitch, eCG, area under the curve, estrus induction, follicular development.

Immunohistochemical detection and localization of sperm-associated antigen 6 (SPAG6) in canine spermatozoa

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The highly conserved mammalian homologue, sperm-associated antigen 6 (SPAG6) is correlated with flagellar motility in mice. Mature SPAG6-deficient male mice are infertile and produce sperm with motility and structural defects. Detection of SPAG6 in canine spermatozoa has not been reported. The objective of this preliminary study was to identify SPAG6 on canine spermatozoa and to determine its localization in fresh semen. The rationale for this study is to determine if SPAG6 can be used as a potential fertility biomarker. From four dogs, the sperm-rich second fractions were collected and pooled. SPAG6 was detected with a standardized immunohistochemistry protocol using a primary SPAG6 monoclonal antibody and biotinylated secondary antibody. Detection and localization was determined via an Olympus microscope under light microscopy (200-600X). For each trial, 100-200 sperm cells were evaluated per slide and a minimum of three slides were counted. The study was repeated three times and the results were confirmed by two individuals. In this preliminary study, in fresh canine spermatozoa, SPAG6 was found only in the midpiece section. This result somewhat differs from what is found in the mouse: localization on SPAG6 at the principal piece, midpiece and head sections. Since it is known that SPAG6-deficient mice have motility and structural defects, using SPAG6 as a fertility biomarker in the canine may prove to be a useful tool for the assessment of canine fertility. Further studies are forth coming to compare detection and localization of SPAG6 in fresh, chilled, and frozen semen.

Keywords: Canine, immunohistochemistry, motility, SPAG6, spermatozoa

Correction of uterine torsion in a 17 year old Belgian mare via bilateral flank laparotomy

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Uterine torsions account for 5-10% of serious obstetric problems in mares[1] and concurrent gastrointestinal complications occur in up to 53% of cases.[2] Determining the etiology of abdominal discomfort in the late gestation mare can be difficult. Diagnosis by transrectal palpation and ultrasonography may be impeded by the fetus.

Uterine torsion must be corrected swiftly and the uterus returned to its normal position for the pregnancy to proceed to term.[3] Prolonged cases may result in uterine rupture, septicemia, or gastrointestinal complications. Methods for correction include rolling, standing flank laparotomy, and ventral midline celiotomy.[2,4,5] The technique chosen is influenced by severity of the mare's pain, fetal viability, uterine size, surgeon's preference, and financial constraints.

A 17 year old Belgian mare was referred for abdominal pain of 12 hours' duration. She was reported to be approximately 10 months in foal at time of presentation. No definitive diagnosis was reached following transrectal palpation and ultrasonography, nor could fetal viability be ascertained. Initial medical management was unsuccessful in relieving the colic and surgical exploration followed. A standing left flank laparotomy was performed and a 360 degree clockwise uterine torsion was diagnosed. A second laparotomy incision was made in the opposite flank to facilitate manipulation and correction of the torsion.

The mare recovered well from surgery, but aborted 48 hours later and retained the placenta. Treatment with uterine lavage and oxytocin over a period of 50 hours resulted in passage of the placenta.

Survival of the mare and foal following uterine torsion requires prompt diagnosis and correction. Differentiation between displacement and uterine torsion may be difficult, and surgery may be required for definitive diagnosis. While a ventral midline approach is preferred in cases when the source of abdominal discomfort is unclear, flank laparotomy may be a viable alternative in cases with financial constraints or high anesthetic risk.

Keywords: Uterine torsion, pregnancy, equine, flank laparotomy, colic.

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Serum anti-sperm antibodies associated with orchitis in a bull

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This report describes the presence of antisperm antibodies (ASA) in a 32-m old Angus bull with orchitis. On presentation, physical and genital system examinations were unremarkable, except for bilaterally heterogeneous ultrasonographic appearance of the testicular parenchyma. Asthenozoospermia (30% motile spermatozoa) and teratozoospermia (26% normal spermatozoa) were noticed, with the main morphologic abnormalities being primary. There was leukospermia and a moderate amount of germ cells in the ejaculate. Differential diagnosis included bilateral orchitis, testicular neoplasia or degeneration with calcinosis. Aerobic semen culture yielded pure growth of alpha hemolytic *Streptococcus sp.*. Testicular biopsy revealed testicular degeneration and interstitial orchitis. Presence of ASA was confirmed by sperm agglutination test and indirect flow cytometry (IgG). Interstitial orchitis is characterized by interstitial lymphocytic infiltration and fibrosis, and can be of infectious or immune origin.[1] Breakdown of the blood-testis barrier during trauma or infection can expose spermatozoa to leukocytes, with formation of ASA.[2] Binding of ASA to spermatozoa can result in sperm agglutination, inhibition of metabolic processes and reduction of sperm motility and velocity.[3] In bulls, ASA can negatively affect sperm capacitation, acrosome reaction and fertility.[2, 3] Treatment was aimed at controlling bacterial growth and inflammation. The bull was treated with flunixin meglumine, ceftiofur sodium and isoniazid for 14 d. Serial semen collections for 2 w remained unchanged, except for resolution of leukospermia within 4 d of the beginning of treatment. Semen and serum were shipped to the VMTH for re-evaluation 18 m after initial presentation. No leukocytes but few germ cells were present. Sperm motility could not be evaluated due to shipping conditions. Sperm morphology improved (48% normal spermatozoa) but was still below the recommended minimum value for classification as a satisfactory breeder. Serum ASA was still detected by agglutination test and indirect flow cytometry. Testicular dysfunction was likely to persist due to testicular degeneration or immune-mediated spermatodysgenesis secondary to orchitis.

Keywords: Antisperm antibodies, bulls, testicular degeneration, autoantibodies, orchitis.

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Granulosa cell tumor in a 12 month-old heifer: clinical and endocrine evaluation

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The presentation (premature mammary development), characterization of inhibin levels, and economic viability of ovarian removal gives this report originality and significance. A 12 month-old maiden Holstein heifer was examined, during a herd reproductive evaluation, for a two-month history of progressive mammary gland development. Differential diagnoses included pregnancy, granulosa cell tumor (GCT), or other endocrine imbalances (i.e., exogenous hormones or cystic ovarian disease). Transrectal palpation ruled out pregnancy, but a large mass craniolateral to the pelvic canal, presumed to be the right ovary, was detected along with an inactive left ovary. A large heteroechogenic multilocular mass in the right ovary was detected via transrectal ultrasound. Serum concentrations of inhibin, testosterone, and progesterone were 11.55 ng/ml, 61.2 pg/mg, and 0.6 ng/ml, respectively, prior to surgery and returned to physiological values within four days after unilateral ovariectomy. The removed ovary weighed 1.66 kg and the GCT was confirmed histopathologically. Cyclicity resumed within 2 weeks and was confirmed by plasma progesterone assays on samples taken every other week. The heifer conceived on the first artificial insemination at 14 months of age. The mammary gland regressed initially, but at 60 days of pregnancy the heifer developed gangrenous mastitis requiring ablation of the right hind quarter. This is the first report describing changes in inhibin concentrations associated with a bovine GCT. The recrudescence of mammary development may have been due to persistently high progesterone after conception. Genetically valuable heifers with similar abnormalities may be better handled as embryo donors after unilateral ovariectomy.

Keywords: Bovine, ovary, granulosa cell tumor, inhibin, neoplasm

Artificial vagina-induced circumferential preputial avulsion in the bovine

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Avulsion of the penis and prepuce of bulls following semen collection with an artificial vagina has negative economic ramifications because of lost semen collections and the potential to induce impotency .[1] Anecdotally, this condition is relatively common, but is likely underreported because the causative trauma is due to poorly trained staff and improper preparation of the artificial vagina. A 3-year old Gelbvieh bull presented following the observation of blood around the sheath after semen collection the week prior to, and also on the day of, presentation. Penile hematomas, penile or preputial lacerations, and preputial avulsions are common reproductive tract injuries in bulls.[2,3] These conditions can result in preputial bleeding and are diagnosed through physical examination. Examination of the penis and prepuce revealed a circumferential preputial avulsion. The penis was exteriorized and mepivacaine (Carbocaine®, 240 mg sc, Hospira, Inc., Lake Forest, IL, USA) was administered proximal, and distal, to the lesion. The injured area was rinsed with sterile saline. Poliglecaprone (Monocryl®, Ethicon, Somerville, NJ, USA) suture (2-0) was utilized to appose the edge of prepenile preputial tissue with that of the glans penis integument. Four cruciate sutures were placed equidistant around the circumference of the penis and simple interrupted sutures were placed between each cruciate. Cefapirin benzathene (Cefa-dri®, 180 mg, Fort Dodge Animal Health, Ft. Dodge, IA, USA) was administered over and under the suture line with a teat cannula, the penis was placed back into the preputial cavity, and an elastic bandage (Elastikon®, Johnson and Johnson, New Brunswick, NJ, USA) was placed on the external prepuce for 5 d to maintain penile retraction. The owner was advised to provide the bull 60 d of sexual rest. Semen collection with an artificial vagina 80 d post-surgery yielded two ejaculates of 7 mL each. The ejaculates possessed 70% and 84% total motility, and 79% and 90% normal morphology. Bulls sustaining a circumferential avulsion of the prepuce and penis can be repaired utilizing the described surgical technique.

Keywords: Bull, prepuce, surgery, artificial vagina, semen collection

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Clitoral Hypertrophy in a Weimaraner

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A 16-month old Weimaraner presented to AUCVM for evaluation of clitoral enlargement and ovariohysterectomy. Upon examination, the bitch was a phenotypic female with a severely enlarged clitoris, which initiated the investigation of a possible reproductive anomaly in this patient. Palpation of the clitoris revealed an os clitoridis and resulted in clitoral engorgement and pelvic thrusting. Pre-surgical blood samples were obtained for hormonal analysis and karyotype, and radiographs were taken to confirm the os clitoridis. During surgery, a uterus was exteriorized and gonads were located in a normal anatomical position consistent with ovaries. Interestingly, neither gonad was contained within an ovarian bursa. Grossly, the right gonad resembled a testis with an attached tubular structure resembling an epididymis; the left gonad more closely resembled an ovary. The uterus and both gonads were removed and submitted for histopathologic examination. The clitoris was surgically removed for cosmetic purposes.

Histologically, the right gonad consisted of numerous seminiferous tubules lined by Sertoli cells, and the interstitial tissue contained Leydig cells. Multiple cross sections of thin walled veins and arterial wall were visualized, suggestive of a pampiniform plexus. The left gonad had identical histologic morphology, but lacked the pampiniform like structure. The uterine horns and body were consistent with normal uterine tissue lined by endometrium.

Pre-surgical testosterone levels of 194.7 pg/ml were consistent with the presence of functional testicular tissue and comparable to that of a cryptorchid male (100-1200 pg/ml).[1] Karyotype was determined to be 78XX and this patient was Sry negative. Normal female karyotype, presence of functional testicular tissue, and lack of concurrent ovarian tissue led to a diagnosis of XX sex reversal.[2] An abnormality of sexual development, XX sex reversal is seen in American Cocker Spaniels[3] and has been reported in 18 other breeds,[4] including the Weimaraner. Investigations into the genetic mechanism leading to Sry negative XX sex reversal have resulted in the exclusion of several possible candidate genes.[5-8] Recent research suggests a novel locus on CFA29 may be responsible [9] for the condition seen in American Cocker Spaniels; however the exact gene or genes responsible for XX sex reversal remain unknown. This case demonstrates the use of diagnostic testing to determine the cause of reproductive anomalies.

Keywords: Sex reversal, genetic defect, clitoris, karyotype

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Management of a Sertoli cell tumor in a stallion

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Equine testicular neoplasms are uncommon,[1] possibly because most males are castrated early in life. Sertoli cell tumors, which arise from nongerminal cells in the seminiferous tubules,[2] have been documented in stallions and are associated with destruction of testicular architecture and contralateral testicular atrophy.[3]

A 23 year old Paso Fino stallion presented for progressive right testicular enlargement of two year's duration, with recently noticed left testicular atrophy. At presentation, ultrasonography showed an enlarged right testis (7.0 x 10.3 x 7.6 cm), with normal echogenicity at the cranial pole and a small, hyperechoic left testis (2.9 x 5.8 x 3.6 cm), with a cranially located circular hypoechoic area. Semen was collected and evaluated to reveal 651million sperm/ejaculate with 30% progressive motility and 50% normal morphology. Multiple testicular biopsy samples were obtained through lateral incisions on both testes. Examination revealed right testicular parenchymal collapse with decreased seminiferous tubule density and few progenitor cells in the cranial pole. Left testicular samples contained dense collagenous tissue consistent with fibrosis and no seminiferous tubules. Neither testis contained detectable spermatozoa. Treatment was postponed until after the 2008 breeding season. In October 2008, the stallion presented for continued right testicular enlargement (10.2 x 12.5 x 9.4 cm) and left testicular atrophy (2.5 x 4.1 x 3.6 cm). Right unilateral castration was performed to preserve potential fertility of the left testis. Examination of the right testis showed an unencapsulated, densely cellular mass replacing normal testicular structures which was histologically consistent with a diffuse Sertoli cell tumor. Four months after surgery, semen evaluation revealed 2.3-3.8 billion sperm/ejaculate with 70-75% progressive motility. Four mares were bred by February 5, 2009 with one pregnancy reported.

Sertoli cell tumors often cause unilateral testicular enlargement with concurrent contralateral atrophy.[3] Descended and retained testes may develop Sertoli cell tumors[1] and metastasis is rare.[3,4] Testicular biopsy is a definitive diagnostic method, but is relatively insensitive. Unilateral castration of the affected testis offers the best prognosis for fertility.[5]

Keywords: Stallion, testes, Sertoi cell tumor, neoplasm,

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